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# (54) TGF-ß RECEPTOR FUSION PROTEIN PHARMACEUTICAL COMPOSITION AND USE THEREOF

(57) Disclosed in the present disclosure are a TGF-β receptor fusion protein pharmaceutical composition and a use thereof. Specifically, the pharmaceutical composition comprises a TGF-β receptor fusion protein in a sodium citrate buffer, and the TGF-β receptor fusion protein comprises a PD-L1 antibody targeting portion and a TGF-βRII extracellular region. In addition, the pharmaceutical composition may also comprise a sugar and a non-ionic surfactant.

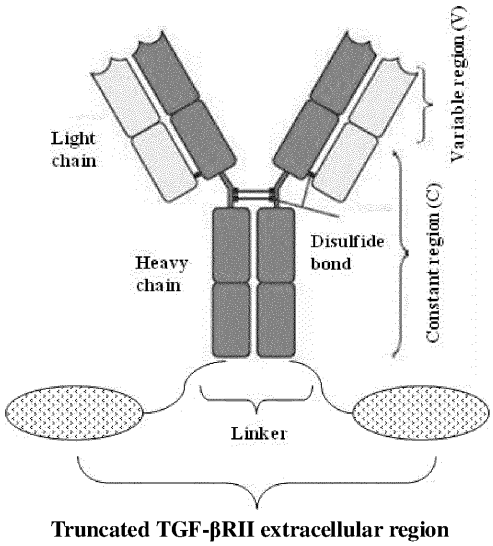


**Figure 1**

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## Description

**[0001]** The present application claims the priority of patent application 201811328326.1 filed on November 9, 2018, which is incorporated herein by reference in its entirety.

## FIELD OF THE INVENTION

**[0002]** The present disclosure belongs to the field of pharmaceutical preparation, and in particular relates to a pharmaceutical composition comprising PD-L1 antibody/TGF-βRII extracellular region fusion protein, and the use thereof as a medicament.

## BACKGROUND OF THE INVENTION

**[0003]** The statements herein only provide background information related to the present disclosure, and do not necessarily constitute the prior art.

**[0004]** During tumor treatment, people have recognized the high toxicity due to chemotherapy, and chemotherapy can lead to the generation of drug-resistant cancer cells. Even if targeted therapies are used, which target over-expressed or over-activated proteins related to tumor survival and growth, there will still be cancer cells that are mutated to reduce or evade the dependence on pathways targeted by the targeted therapy, and those cancer cells would survive via other pathways.

**[0005]** Tumor immunotherapy has attracted much attention in recent years, and is the focus in the field of tumor treatment. The outstanding advantage of such therapy is the increased difficulty in generating drug resistance. Tumor immunotherapy mainly uses immunological principles and methods to improve the immunogenicity of tumor cells and the sensitivity to effector cell killing, and to stimulate and enhance the anti-tumor immune response in organism. Tumor immunotherapy involves the infusion of immune cells and effector molecules into a host, and these two cooperate with the immune system to kill tumors and inhibit tumor growth in organism.

**[0006]** Programmed death receptor 1 (PD-1) is a member of the CD28 superfamily. PD-1 is expressed on activated T cells, B cells and myeloid cells. PD-1 has two ligands, programmed death ligand 1 (PD-L1) and PD-L2. PD-L1 interacts with the receptor PD-1 on T cells, and plays an important role in the negative regulation of immune response. The expression of PD-L1 protein can be detected in many human tumor tissues. The microenvironment at the tumor site can induce the expression of PD-L1 on tumor cells, and the expressed PD-L1 in turn contributes to the tumorigenesis and growth, and induces the apoptosis of anti-tumor T cells. The inhibitors of PD-1/PD-L1 pathway block the binding of PD-1 to PD-L1, block negative regulatory signals, restore T cell activity, and enhance immune response. Therefore, immunomodulation with PD-1/PD-L1 as the target is of great significance to tumor suppression.

**[0007]** Transforming growth factor-β (TGF-β) belongs to the TGF-β superfamily that regulates cell growth and differentiation. TGF-β transmits signals through a heterotetrameric receptor complex, which is composed of two type I and two type II transmembrane serine/threonine kinase receptors.

**[0008]** TGF-β is a multifunctional cytokine, which exerts a tumor-suppressing or tumor-promoting effect in a cell-dependent or background-dependent manner. The tumor-suppressing effect of TGF-β depends on the ability to induce the expression of multiple genes. When mutations or epigenetic modifications are introduced during tumor development, cancer cells are gradually tolerant to the inhibitory effect of TGF-β, which ultimately leads to tumor development.

**[0009]** Studies have found that blocking the TGF-β signaling pathway can reduce tumor metastasis. It was found that the metastasis ability of tumor cells was inhibited when the TGF-β signaling pathway of breast tumor cell lines was inhibited by the truncated Smad2/3 negative mutant. The study of the instability of colon cancer microsatellite found that the inactive mutation of TGF-βRII reduced metastasis and increased the postoperative survival rate of patients. However, in general, the effect is weak when inhibitor of TGF-β signaling pathway is administered alone in clinical treatment, probably because TGF-β is mainly abnormally expressed in tumor cells, whereas it is difficult for the inhibitor of TGF-β signaling pathway alone to target tumor, resulting in low efficacy or low bioavailability of the inhibitor.

**[0010]** Therefore, on the basis of targeting and neutralizing TGF-β in a tumor microenvironment, inhibiting the PD-1/PD-L1 pathway can restore the activity of T cells, enhance the immune response, and improve the inhibiting effect of tumorigenesis and development more effectively.

**[0011]** A previous PCT application of the applicant PCT/CN2016/104320 (publication number WO2017084495) provides a PD-L1 antibody. Antibody/TGF-β receptor fusion protein has been published at present, such as in WO2006074451A2, WO2009152610A1, WO2011109789A2, WO2013164694A1, WO2014164427A1, WO2015077540A2, WO9309228A1, WO9409815A1, WO2015077540A2, WO2015118175A2, etc. Among them, Merck discloses a PD-L1/TGF-β bifunctional fusion protein Bintrafusp Alfa (WO2015118175, also known as M7824, FP17022). Currently, Bintrafusp Alfa has been in clinical phase of tumor diseases such as gastric cancer, lung cancer, esophageal cancer, NSCLC, biliary cancer. However, the antibody medicaments in the prior art become unstable due to large

molecular weights, complex structures, and being susceptible to degradation, polymerization or occurrence of undesirable chemical modifications. In order to make the antibody suitable for administration, maintain stability during storage and subsequent use, and to exert a better effect, the research on stable preparations of antibody medicaments is particularly important.

## SUMMARY OF THE INVENTION

**[0012]** The present disclosure provides a pharmaceutical composition comprising a PD-L1/TGF-βRII fusion protein, which is more conducive to production and administration, and is more stable in performance; the pharmaceutical composition comprises:

- a TGF-β receptor fusion protein, and
- a buffer,

wherein the buffer is selected from the group consisting of a histidine salt buffer, a succinate buffer, a phosphate buffer and a citrate buffer.

**[0013]** In some embodiments, the buffer is a citrate buffer. In some embodiments, the histidine salt buffer is histidine-hydrochloric acid buffer; and the succinate buffer is succinic acid-sodium succinate buffer; the citrate buffer is citric acid-sodium citrate buffer; In some embodiments, the buffer is citric acid-citrate sodium buffer.

**[0014]** In an alternative embodiment, the concentration of the TGF-β receptor fusion protein in the pharmaceutical composition described above is about 0.5 mg/ml to about 100 mg/ml, preferably about 30 mg/ml to about 70 mg/ml.

**[0015]** In some embodiments, the concentration of the TGF-β receptor fusion protein in the pharmaceutical composition is 0.5 mg/ml to 100 mg/ml, preferably 30 mg/ml to 70 mg/ml. The non-limiting examples of the concentration of TGF-β receptor fusion protein involve: about 30mg/ml, about 35mg/ml, about 40mg/ml, about 45mg/ml, about 50mg/ml, about 55mg/ml, about 60mg/ml, about 65mg/ml, about 70mg/ml, preferably about 50mg/ml.

**[0016]** In some embodiments, the concentration of the TGF-β receptor fusion protein in the pharmaceutical composition is 30mg/ml, 35mg/ml, 40mg/ml, 45mg/ml, 50mg/ml, 55mg/ml, 60mg/ml, 65mg/ml, 70 mg/ml, more preferably 50 mg/ml.

**[0017]** In an alternative embodiment, the pH value of the buffer in the pharmaceutical composition described above is about 5.0 to about 7.5, preferably about 6.0 to about 6.5, and optionally about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, more preferably about 6.2.

**[0018]** In some embodiments, the pH value of the buffer is 5.0 to 7.5, or 6.0 to 6.5, preferably 6.0, 6.1, 6.2, 6.3, 6.4 or 6.5, more preferably 6.2.

**[0019]** In an alternative embodiment, the concentration of the buffer is about 5mM to about 30mM, preferably about 5mM to about 20mM; non-limiting examples thereof involve 5mM, 6mM, 7mM, 8mM, 9mM, lOmM, 12mM, 14mM, 16mM, 18mM, 20mM, more preferably 10mM.

**[0020]** In some embodiments, the concentration of the buffer is 5 mM to 30 mM, preferably 5 mM to 20 mM; and in some embodiments, the concentration of the buffer is about 10 mM, about 12 mM, about 14 mM, about 16 mM, about 18 mM, about 20 mM, and more preferably about 10 mM.

**[0021]** In an alternative embodiment, the pharmaceutical composition described above also comprises saccharide. The "saccharide" in the present disclosure comprises conventional compounds/compositions $(CH_2O)_n$ or derivatives thereof, comprising monosaccharides, disaccharides, trisaccharides, polysaccharides, sugar alcohols, reducing saccharides, non-reducing saccharides, and the like. In some embodiments, the saccharide is selected from the group consisting of: glucose, sucrose, trehalose, lactose, fructose, dextran, glycerol, erythritol, glycerol, arabitol, xylitol, sorbitol, mannitol, melibiose, melezitose, melitriose, mannotriose, stachyose, maltose, lactulose, maltulose, sorbitol, maltitol, lactitol, iso-maltulose and so on. The preferred saccharide is a non-reducing disaccharide, more preferably trehalose or sucrose, and most preferably sucrose.

**[0022]** In an alternative embodiment, the concentration of the saccharide in the pharmaceutical composition described above is about 50 mg/ml to about 100 mg/ml, preferably about 60 mg/ml to about 90 mg/ml; non-limiting examples involve 60 mg/ml, 65 mg/ml, 70mg/ml, 75mg/ml, 80mg/ml, 85mg/ml, 90mg/ml, most preferably 80mg/ml.

**[0023]** In some embodiments, the concentration of the saccharide is 50 mg/ml to 100 mg/ml, preferably 60 mg/ml to 90 mg/ml; and in some embodiments, the concentration of the saccharide is about 60 mg/ml, about 65 mg/ml, about 70 mg/ml, about 75mg/ml, about 80mg/ml, about 85mg/ml or about 90mg/ml.

**[0024]** In an alternative embodiment, the pharmaceutical composition described above further comprises a surfactant, which may be selected from the group consisting of polysorbate 20, polysorbate 80, polyhydroxyalkylene, Triton, sodium dodecyl sulfonate, sodium lauryl sulfonate, sodium octyl glycoside, lauryl-sulfobetaine, myristyl-sulfobetaine, linoleyl-sulfobetaine, stearyl-sulfobetaine, lauryl-sarcosine, myristyl-sarcosine, linoleyl-sarcosine, stearyl-sarcosine, linoleyl-betaine, myristyl-betaine, cetyl-betaine, laurel amidopropyl-betaine, cocaamidopropyl-betaine, linoleamidopropyl-betaine, myristamidopropyl-betaine, palmitamidopropyl-betaine, isostearyl amidopropyl-betaine, myristamidopropyl-

dimethylamine, palmamidopropyl-dimethylamine, isostearamidopropyl-dimethylamine, sodium methyl cocoyl, sodium methyl oleyl taurate, polyethylene glycol, polypropylene glycol, copolymer of ethylene and propylene glycol, etc. The preferred surfactant is polysorbate 80 or polysorbate 20, more preferably polysorbate 80.

**[0025]** In another alternative embodiment, the concentration of the surfactant in the pharmaceutical composition described above is about 0.1 mg/ml to about 0.8 mg/ml, more preferably about 0.4 mg/ml to about 0.8 mg/ml. In some embodiments, the concentration of the surfactant is 0.1mg/ml to 0.8mg/ml, preferably 0.4mg/ml to 0.8mg/ml, more preferably about 0.4mg/ml, about 0.45mg/ml, about 0.5mg/ml, about 0.55mg/ml, about 0.6mg/ml, about 0.7mg/ml, about 0.8mg/ml.

**[0026]** In some embodiments, the concentration of the surfactant is 0.4mg/ml, 0.45mg/ml, 0.5mg/ml, 0.55mg/ml, 0.6mg/ml, 0.7mg/ml or 0.8mg/ml, more specifically 0.4mg/ml.

**[0027]** In an alternative embodiment, the pharmaceutical composition described above comprises:
(a) about 0.5mg/ml to about 100mg/ml TGF-β receptor fusion protein, (b) about 5mM to about 30mM citrate buffer, (c) about 50mg/ml to about 100mg/ml sucrose, and (d) about 0.1 mg/ml to about 0.8 mg/ml polysorbate 80, preferably the pH of the pharmaceutical composition is about 5.0 to about 7.5, more preferably about 6.0 to about 6.5.

**[0028]** In an alternative embodiment, the pharmaceutical composition described above comprises:

| | |
|---|---|
| 0.5mg/ml to 100mg/ml | TGF-β receptor fusion protein |
| 5mM to 30mM | citrate buffer |
| 50mg/ml to 100mg/ml | sucrose, and |
| 0.1mg/ml to 0.8mg/ml | polysorbate 80; |

preferably, the pH of the pharmaceutical composition is 5.0 to 7.5, more preferably 6.0 to 6.5.

**[0029]** In an alternative embodiment, the pharmaceutical composition described above comprises:
(a) about 30mg/ml to about 70mg/ml TGF-β receptor fusion protein, (b) about 5mM to about 20mM citric acid-sodium citrate buffer, (c) about 60mg/ml to about 90mg/ml sucrose, and (d) about 0.4 mg/ml to about 0.8 mg/ml polysorbate 80, preferably, the pH of the pharmaceutical composition is about 6.0 to about 6.5.

**[0030]** In an alternative embodiment, the pharmaceutical composition described above comprises:

| | |
|---|---|
| 30mg/ml to 70mg/ml | TGF-β receptor fusion protein |
| 5mM to 20mM | citric acid-sodium citrate buffer |
| 60mg/ml to 90mg/ml | sucrose, and |
| 0.4mg/ml to 0.8mg/ml | polysorbate 80; |

the pH of the pharmaceutical composition is about 6.0 to about 6.5.

**[0031]** In an alternative embodiment, the pharmaceutical composition comprises:
(a) about 50mg/ml TGF-β receptor fusion protein, (b) about lOmM citric acid-sodium citrate buffer, (c) about 80mg/ml sucrose, and (d) about 0.4mg/ml polysorbate 80, the pH of the pharmaceutical composition is preferably about 6.2.

**[0032]** In an alternative embodiment, the pharmaceutical composition comprises:

| | |
|---|---|
| 50mg/ml | TGF-β receptor fusion protein |
| 10mM | citric acid-sodium citrate buffer |
| 80mg/ml | sucrose, and |
| 0.4mg/ml | polysorbate 80; |

preferably, the pH of the pharmaceutical composition is about 6.2.

**[0033]** In an alternative embodiment, the TGF-β receptor fusion protein in the pharmaceutical composition described above is shown as general formula (I):

Ab-L-TGF-βRII ECD (I)

wherein, the TGF-βRII ECD is a truncated form of an extracellular region of TGF-βRII;
Ab is a PD-L1 antibody or antigen-binding fragment thereof;
L is a linker sequence.

**[0034]** In an alternative embodiment, the linker sequence in the pharmaceutical composition described above is $(G_4S)_xG$, wherein x is an integer of 3-6. In an alternative embodiment, x is 3, 4, 5 or 6, preferably 4.

**[0035]** In an alternative embodiment, the truncated form of the extracellular region of TGF-βRII is a sequence of TGF-βRII extracellular domain (shown as SEQ ID NO: 14) with a deletion of at most 26 consecutive amino acid residues at amino terminus (also referred as N terminus). In some embodiments, the truncated form of the extracellular region of TGF-βRII is a sequence of TGF-βRII extracellular domain with a deletion of 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26 consecutive amino acid residues at N terminus. In some embodiments, the sequence of the TGF-βRII ECD in the pharmaceutical composition described above is shown as SEQ ID NO: 14, 15, 16 or 17; preferably, the sequence shown as SEQ ID NO: 15.

**[0036]** In an alternative embodiment, the PD-L1 antibody or antigen-binding fragment thereof in the pharmaceutical composition described above comprises:

HCDR1, HCDR2 and HCDR3 shown as SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3, respectively; and
LCDR1, LCDR2 and LCDR3 shown as SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6, respectively.

**[0037]** In an alternative embodiment, the PD-L1 antibody or antigen-binding fragment thereof in the pharmaceutical composition described above comprises:

HCDR1, HCDR2 and HCDR3 shown as SEQ ID NO: 1, SEQ ID NO: 10 and SEQ ID NO: 3, respectively, and
LCDR1, LCDR2 and LCDR3 shown as SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6, respectively.

**[0038]** In an alternative embodiment, the PD-L1 antibody or antigen-binding fragment thereof in the pharmaceutical composition described above comprises:

a heavy chain variable region shown as SEQ ID NO: 7 and
a light chain variable region shown as SEQ ID NO: 8;

or, comprises:

a heavy chain variable region shown as SEQ ID NO: 9 and
a light chain variable region shown as SEQ ID NO: 11.

**[0039]** In an alternative embodiment, the heavy chain amino acid sequence of the PD-L1 antibody in the pharmaceutical composition described above is shown as SEQ ID NO: 12 or has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the amino acid sequence shown as SEQ ID NO: 12; the light chain amino acid sequence of the PD-L1 antibody is shown as SEQ ID NO: 13 or has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the amino acid sequence shown as SEQ ID NO: 13.

**[0040]** In an alternative embodiment of the pharmaceutical composition described above, in the TGF-β receptor fusion protein, the TGF-βRII ECD is fused to the carboxyl terminus of the PD-L1 antibody heavy chain through a linker sequence.

**[0041]** In some embodiments, the TGF-β receptor fusion protein comprises:

- a fusion peptide formed by the heavy chain of the PD-L1 antibody fused to TGF-βRII ECD, the sequence of which is shown as SEQ ID NO: 23 or has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the sequence shown as SEQ ID NO: 23, and
- the light chain of the PD-L1 antibody, the sequence of which is shown as SEQ ID NO: 13 or has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the sequence shown as SEQ ID NO: 13.

**[0042]** In other embodiments, the TGF-β receptor fusion protein comprises:

- a fusion peptide formed by the heavy chain of the PD-L1 antibody fused to TGF-βRII ECD, the sequence of which is shown as SEQ ID NO: 24 or has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the sequence shown as SEQ ID NO: 24, and
- the light chain of the PD-L1 antibody, the sequence of which is shown as SEQ ID NO: 13 or has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the sequence shown as SEQ ID NO: 13.

**[0043]** The present disclosure also provides a method for preparing the pharmaceutical composition described above, which comprises a step of contacting TGF-β receptor fusion protein with a buffer, for example, performing buffer replacement on the TGF-β receptor fusion protein stock solution, and the buffer is preferably citrate buffer; more preferably citric acid-sodium citrate buffer, the concentration of the buffer is preferably about 5 mM to about 20 mM; the non-limiting examples involve 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, lOmM, 12mM, 14mM, 16mM, 18mM, 20mM, more preferably 10mM; the pH of the buffer is about 6.0 to about 6.5, the non-limiting examples involve 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, preferably 6.2. In an alternative embodiment, the concentration of the buffer is 5 mM to 20 mM, the non-limiting examples involve about 5mM, about 6mM, about 7mM, about 8mM, about 9mM, about lOmM, about 12mM, about 14mM, about 16mM, about 18mM, about 20mM, more preferably about lOmM; the pH of the buffer is 6.0 to 6.5, the non-limiting examples involve about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, preferably about 6.2.

**[0044]** The present disclosure also provides a method for preparing the pharmaceutical composition described above, further comprising the following steps after contacting the TGF-β receptor fusion protein with the buffer: adding sucrose and polysorbate 80 to the obtained solution (no precedence order between the two), and then adjusting the volume with the buffer, wherein the concentration of the buffer solution is preferably about 5mM to about 20mM, more preferably 5mM to 20mM, the non-limiting examples involve 5mM, 8mM, lOmM, 12mM, 14mM, 16mM, 18mM, 20mM; the pH of the buffer is about 6.0 to about 6.5, the non-limiting examples involve 6.0, 6.1, 6.2, 6.3, 6.4, 6.5.

**[0045]** The present disclosure also provides a method for preparing a lyophilized preparation comprising TGF-β receptor fusion protein, which comprises a step of lyophilizing the pharmaceutical composition described above.

**[0046]** In an alternative embodiment, the method for preparing a lyophilized preparation described above comprising the TGF-β receptor fusion protein, wherein the lyophilization is performed according to a method known in the art, such as but not limited to steps comprising pre-freezing, primary drying and secondary drying. The skilled persons understand that any method for removing water from the pharmaceutical composition in the present disclosure is applicable to the present disclosure.

**[0047]** The present disclosure also provides a lyophilized preparation comprising the TGF-β receptor fusion protein, which is prepared by the method for preparing a lyophilized preparation described above.

**[0048]** The present disclosure also provides a lyophilized preparation comprising the TGF-β receptor fusion protein, which can be reconstituted to form the pharmaceutical composition described above.

**[0049]** In some embodiments, the lyophilized preparation can be stable at 2°C to 8°C for at least 3 months, at least 6 months, at least 12 months, at least 18 months, or at least 24 months. In some embodiments, the lyophilized preparation can be stable at 40°C for at least 7 days, at least 14 days, or at least 28 days.

**[0050]** The present disclosure also provides a reconstituted solution comprising the TGF-β receptor fusion protein, which is obtained by re-reconstituting the lyophilized preparation comprising TGF-β receptor fusion protein described above.

**[0051]** The present disclosure also provides a method for preparing the reconstituted solution comprising the TGF-β receptor fusion protein described above, which comprises: a step of re-reconstituting the lyophilized preparation described above, the solution used for reconstitution comprises, but is not limited to, water for injection, physiological saline or glucose solution, preferably water for injection.

**[0052]** The present disclosure further provides an article of manufacture or kit, comprising: the pharmaceutical composition according to the present disclosure; and container(s).

**[0053]** In some embodiments, the container is a glass bottle, such as but not limited to, an injection bottle made of neutral borosilicate glass vial.

**[0054]** The present disclosure also provides an article of manufacture, comprising container(s), which comprise(s) the pharmaceutical composition described above, or the lyophilized preparation thereof, or a reconstituted solution of the lyophilized preparation.

**[0055]** The present disclosure also provides the use of any one selected from the following in the preparation of a medicament:

the pharmaceutical composition described above, or the lyophilized preparation, or the reconstituted solution of the lyophilized preparation, or the article of manufacture; the medicament is used to treat or inhibit disease(s) or disorder(s) of tumor cell proliferation or metastasis.

**[0056]** In some embodiments, the disease(s) or disorder(s) is/are tumor.

**[0057]** In some embodiments, the disease(s) or disorder(s) is/are selected from the group consisting of: colorectal cancer, breast cancer, ovarian cancer, pancreatic cancer, gastric cancer, prostate cancer, kidney cancer, cervical cancer, myeloma, lymphoma, leukemia, thyroid cancer, endometrial cancer, uterine cancer, bladder cancer, neuroendocrine cancer, head and neck cancer, liver cancer, nasopharyngeal carcinoma, testicular cancer, lung cancer, small cell lung cancer, non-small cell lung cancer, melanoma, basal cell cutaneous carcinoma, squamous cell cutaneous carcinoma, dermatofibrosarcoma protuberans, Merkel cell carcinoma, glioblastoma, glioma, sarcoma, mesothelioma, and myelodysplastic syndrome.

**[0058]** The present disclosure also provides a method for treating or inhibiting disease(s) or disorder(s) related to

proliferation or metastasis of cancer cell, comprising providing a therapeutically effective amount of the pharmaceutical composition described above or the lyophilized preparation, or the reconstituted solution, or the article of manufacture, to a subject in need. In some embodiments, the method comprises administering to the subject a unit dose of composition comprising: 0.1 mg to 3000mg of the TGF-β receptor fusion protein as described above, the pharmaceutical composition, or the lyophilized preparation, or the reconstituted solution, or the article of manufacture. In some embodiments, the disease(s) or disorder(s) is/are tumor. In some embodiments, the disease(s) or disorder(s) is/are selected from the group consisting of: colorectal cancer, breast cancer, ovarian cancer, pancreatic cancer, gastric cancer, prostate cancer, kidney cancer, cervical cancer, myeloma, lymphoma, leukemia, thyroid cancer, endometrial cancer, uterine cancer, bladder cancer, neuroendocrine cancer, head and neck cancer, liver cancer, nasopharyngeal carcinoma, testicular cancer, lung cancer, small cell lung cancer, non-small cell lung cancer, melanoma, basal cell cutaneous carcinoma, squamous cell cutaneous carcinoma, dermatofibrosarcoma protuberans, Merkel cell carcinoma, glioblastoma, glioma, sarcoma, mesothelioma, and myelodysplastic syndrome.

**[0059]** The present invention also provides the TGF-β receptor fusion protein, pharmaceutical composition, or lyophilized preparation, or reconstituted solution, or article of manufacture described above, for treating or inhibiting disease(s) or disorder(s) related to proliferation or metastasis of cancer cell. In some embodiments, the disease(s) or disorder(s) is/are tumor. In some embodiments, the disease(s) or disorder(s) is/are selected from the group consisting of: colorectal cancer, breast cancer, ovarian cancer, pancreatic cancer, gastric cancer, prostate cancer, kidney cancer, cervical cancer, myeloma, lymphoma, leukemia, thyroid cancer, endometrial cancer, uterine cancer, bladder cancer, neuroendocrine cancer, head and neck cancer, liver cancer, nasopharyngeal carcinoma, testicular cancer, lung cancer, small cell lung cancer, non-small cell lung cancer, melanoma, basal cell cutaneous carcinoma, squamous cell cutaneous carcinoma, dermatofibrosarcoma protuberans, Merkel cell carcinoma, glioblastoma, glioma, sarcoma, mesothelioma, and myelodysplastic syndrome.

**[0060]** As is well known to those skilled in the art, one, some or all of the features of the various embodiments described in the present disclosure can be further combined to form other embodiments of the present disclosure. The above embodiments of the present disclosure and other embodiments obtained by combination are further illustrated by the following detailed description.

## DESCRIPTION OF THE DRAWINGS

**[0061]**

Figure 1: Schematic diagram showing the structure of the fusion protein.

Figure 2: Results showing the binding of fusion proteins to human TGF-β1 *in vitro.*

Figure 3: Results showing the binding of fusion proteins to human TGF-β1 *in vitro.*

Figure 4: Results showing the binding of fusion proteins to human PD-L1 *in vitro.*

Figure 5: Result showing the detection of PD-1/PD-L1 pathway blocking by fusion proteins *in vitro.*

Figure 6: Fusion proteins inhibit TGFβ-induced activity of pSMAD3 reporter in a dose-dependent manner.

Figure 7: All samples of fusion proteins enhance the secretion of the cytokine IFN-γ by activated T lymphocytes.

Figure 8: Effect of fusion proteins on tumor weight of tumor-bearing mice.

## DETAILED DESCRIPTION OF THE INVENTION

### Terminology

**[0062]** For the disclosure to be more readily understood, certain technical and scientific terms are specifically defined below. Unless specifically defined herein, all other technical and scientific terms used herein have the meaning commonly understood by one of ordinary skills in the art to which this disclosure pertains.

**[0063]** "Buffer" refers to a solution that is tolerated to the change of pH through the action of acid-base conjugate components. Examples of buffers that can control the pH within an appropriate range include acetate, succinate, gluconate, histidine, oxalate, lactate, phosphate, citrate, tartrate, fumarate and glycylglycine.

**[0064]** "Histidine salt buffer" is a buffer comprising histidine radical ions. Examples of histidine salt buffers include

histidine-hydrochloride, histidine-acetate, histidine-phosphate, histidine-sulfate, and the like; preferably histidine-hydrochloride buffer. Histidine-hydrochloride buffer is prepared from histidine and hydrochloric acid.

**[0065]** "Citrate buffer" is a buffer that comprises citrate radical ions. Examples of the citrate buffers include citric acid-sodium citrate, citrate-potassium citrate, citrate-calcium citrate, citrate-magnesium citrate, and the like. The preferred citrate buffer is citric acid-sodium citrate.

**[0066]** "Succinate buffer" is a buffer that comprises succinate radical ions. Examples of the succinate buffers include succinic acid -sodium succinate, succinic acid -potassium succinate, succinic acid -succinate calcium, and the like. The preferred succinate buffer is succinic acid -sodium succinate.

**[0067]** "Phosphate buffer" is a buffer that comprises phosphate radical ions. Examples of the phosphate buffers include disodium hydrogen phosphate-sodium dihydrogen phosphate, disodium hydrogen phosphate-potassium dihydrogen phosphate, and the like. The preferred phosphate buffer is disodium hydrogen phosphate-sodium dihydrogen phosphate.

**[0068]** "Acetate buffer" is a buffer comprising acetate radical ions. Examples of acetate buffers include acetic acid-sodium acetate, histidine acetate, acetic acid-potassium acetate, acetic acid-calcium acetate, acetic acid-magnesium acetate, and the like. The preferred acetate buffer is acetic acid-sodium acetate.

**[0069]** "Pharmaceutical composition" refers to a mixture comprising one or more of the compounds described herein or the physiologically/pharmaceutically acceptable salts or prodrugs thereof and other chemical components, such as physiologically/pharmaceutically acceptable carrier(s) and excipient(s). The purpose of the pharmaceutical composition is to maintain the stability of the active ingredient antibody, to promote the administration to the organism, and facilitate the absorption of the active ingredient as to exert the biological activity. The "pharmaceutical composition" and "preparation" used herein are not mutually exclusive.

**[0070]** Unless otherwise specified, when referring to the solution form of the pharmaceutical composition described in the present disclosure, the solvent therein is water.

**[0071]** "Lyophilized preparation" refers to a preparation or a pharmaceutical composition obtained after a step of lyophilizing (for example, a vacuum freeze-drying step) the pharmaceutical composition in its liquid or solution form, or lyophilizing the preparation in its liquid or solution form.

**[0072]** The term "about" or "approximately" as used in the present disclosure means that the value is within an acceptable error range of the specific value determined by the skilled persons ordinary in the art, and the value depends partially on how it is measured or determined (i.e., the limit of the measuring system). For example, "about" or "approximately" in the art refers to a standard deviation less than one or more than one. Alternatively, "about" or "approximately" or "substantially comprising" refers to a range up to 20%. In addition, particularly for biological systems or processes, the term means an order of magnitude up to one, or up to 5 times higher than the value. Unless otherwise specified, the meaning of "about XX" or "approximately XX" or "substantially comprising XX" used in present disclosure refers to a value within an acceptable error range of the specific value "XX" (including the value "XX" itself, as well as values within an acceptable error range of the value as determined by the skilled persons ordinary in the art).

**[0073]** The pharmaceutical composition described in the present disclosure is capable of achieving a stable effect: the TGF-β receptor fusion protein or the pharmaceutical composition thereof substantially retains the physical stability and/or chemical stability and/or biological activity after storage; preferably, the pharmaceutical composition substantially retains the physical and chemical stability and its biological activity after storage. The shelf life is generally determined based on the predetermined shelf life of the pharmaceutical composition. There are currently many analytical techniques for measuring the stability of active ingredients, which can measure the stability after storage at a given temperature for a given period of time.

**[0074]** A stable pharmaceutical preparation of antibody or protein is such preparation for which no significant changes are observed under the following conditions: being stored at a refrigerated temperature (2-8°C) for at least 3 months, preferably for 6 months, more preferably for 1 year, and even more preferably up to 2 years. In addition, stable liquid preparations include liquid preparations that exhibit desired characteristics after being stored at a temperature (including 25°C) for 1 month, 3 months, 6 months, or stored at 40°C for a period of 28 days.

**[0075]** Typical acceptable standards for stability are as follows: as measured by SEC-HPLC, usually no more than about 10%, preferably no more than about 5% of the active ingredients (such as proteins, antibodies) are degraded. By visual inspection, the pharmaceutical preparation is pale yellow nearly colorless, clear or colorless liquid, or clear to slightly milky white, or pale yellow nearly colorless clear liquid. The change of concentration, pH and osmolality of the preparation is no more than ±10%. A truncation of no more than about 10%, preferably no more than about 5% is generally observed. Usually no more than about 10%, preferably no more than about 5% of aggregates are formed.

**[0076]** The active ingredient in the pharmaceutical preparation is deemed to "retain its physical stability", if the antibody does not show any significant increase in aggregation, precipitation and/or denaturation by visual inspection of color and/or clarity, or UV light scattering, size exclusion chromatography (SEC) and dynamic light scattering (DLS). Changes in protein conformation can be evaluated by fluorescence spectroscopy (which determines the tertiary structure of the protein) and by FTIR spectroscopy (which determines the secondary structure of the protein).

**[0077]** The active ingredient (such as protein or antibody) in the pharmaceutical preparation is deemed to "retain its

chemical stability", if the active ingredient (such as protein or antibody) does not show any significant chemical change. By detecting and quantifying chemically altered forms of proteins or antibodies, chemical stability can be assessed. Degradation processes that often lead to a change of chemical structure of proteins include hydrolysis or truncation (evaluated by methods such as size exclusion chromatography and SDS-PAGE), oxidation (evaluated by methods such as peptide mapping combined with mass spectrometry or MALDI/TOF/MS, etc.), deamidation (evaluated by methods such as ion exchange chromatography, capillary isoelectric focusing, peptide mapping, measurement of isoaspartic acid content, etc.) and isomerization (evaluated by measurement of isoaspartic acid content, peptide mapping etc.).

**[0078]** An active ingredient (e.g. protein or antibody) "retains its biological stability" in the pharmaceutical preparation, if the active ingredient (e.g. protein or antibody), for a given period of time, exhibits a biological activity within a predetermined range of that when the pharmaceutical formulation is prepared. The biological activity of an active ingredient (such as a protein or antibody) can be determined, for example, by antigen-binding assay.

**[0079]** As used in the disclosure, the three-letter code and the single-letter code for amino acids are as described in J. Biol. Chem, 243, p3558 (1968).

**[0080]** As used in the present disclosure, "antibody" refers to immunoglobulin, a four-peptide chain structure formed by two identical heavy chains and two identical light chains connected by inter-chain disulfide bond(s).

**[0081]** In the present disclosure, the antibody light chain described in the present disclosure further comprises light chain constant region(s), which comprise(s) a human or murine κ, λ chain or a variant(s) thereof.

**[0082]** In the present disclosure, the antibody heavy chain described in the present disclosure further comprises heavy chain constant region(s), which comprise(s) a human or murine IgG1, IgG2, IgG3, IgG4 or variant(s) thereof.

**[0083]** At the N-terminus of the antibody heavy chain and light chain, a region of about 110 amino acids varies largely, which is known as variable region (Fv region); the amino acid sequence at the C-terminus is relatively stable, which is known as constant region. Variable region comprises three hypervariable regions (HVR) and four FR regions (FR) with relatively conserved sequence. Three hypervariable regions determine the specificity of an antibody, also known as complementarity determining region (CDR). Each light chain variable region (LCVR or VL) and each heavy chain variable region (HCVR or VH) is composed of three CDR regions and four FR regions, arranged from the amino terminus to the carboxyl terminus as following: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Three light chain CDR regions refer to LCDR1, LCDR2, and LCDR3; three heavy chain CDR regions refer to HCDR1, HCDR2 and HCDR3. The number and location of CDR region amino acid residues in LCVR and HCVR regions of the antibody or the antigen binding fragment herein comply with known Kabat numbering criteria (LCDR1-3, HCDR1-3), or comply with Kabat and Chothia numbering criteria; Kabat numbering criteria (see Kabat et al (1991), Sequences of Proteins of Immunological Interest, the 5th edition, Public Health Service, National Institutes of Health, Bethesda, MD), and Chothia numbering criteria (see Al-Lazikani et al (1997) JMB 273: 927-948).

**[0084]** The antibody of the present disclosure involves murine antibody, chimeric antibody and humanized antibody, preferably humanized antibody.

**[0085]** As used in the present disclosure, "the antibody or the binding fragment thereof" or "functional fragment" refers to Fab fragment, Fab' fragment, F(ab')2 fragment having antigen-binding activity, as well as Fv fragment, scFv fragment binding to antigen. Fv fragment is the minimum antibody fragment which comprises all antigen-binding sites, Fv fragment comprises a heavy chain variable region and a light chain variable region, but without constant region(s). Generally, Fv antibody further comprises a polypeptide linker between the VH and VL domains to form a structure required for antigen-binding. Also, different linkers can be used to connect the variable regions of two antibodies to form a polypeptide chain, named single chain antibody or single chain Fv (sFv). As used in the present disclosure, the term "binding with PD-L1" means the ability to interact with human PD-L1. As used in the present disclosure, the term "antigen-binding site" refers to inconsecutive or consecutive three-dimensional sites on an antibody or on antigen-binding fragment thereof, which recognize a target antigen and specifically bind to the antigen.

**[0086]** The term "murine antibody" in the present disclosure refers to anti-human PD-L1 monoclonal antibody prepared according to the knowledge and skills in the field. During the preparation, test subject is injected with PD-L1 antigen, and then hybridoma expressing antibody which possesses desired sequence or functional characteristics is isolated.

**[0087]** The term "chimeric antibody" is an antibody which is formed by fusing the variable region of a non-human (such as murine) antibody with the constant region of human antibody, so as to alleviate the non-human (such as murine) antibody-induced immune response. To establish a chimeric antibody, a hybridoma secreting specific monoclonal antibody is established firstly, then genes of variable region are cloned from hybridoma cells, and then genes of constant region of human antibody are cloned as desired, the genes of non-human (such as murine) antibody variable region are ligated with genes of human constant region to form a chimeric gene which can be inserted into a human vector, and the chimeric antibody molecule is finally expressed in a eukaryotic or prokaryotic industrial system. In a preferred embodiment of the present disclosure, the light chain of the PD-L1 chimeric antibody further comprises light chain constant region(s) derived from human κ, λ chain or variant(s) thereof. The heavy chain of PD-L1 chimeric antibody further comprises heavy chain constant region(s) derived from human IgG1, IgG2, IgG3, IgG4 or variant(s) thereof. The constant region(s) of human antibody can be selected from heavy chain constant region(s) derived from human IgG1, IgG2, IgG3,

IgG4 or variant(s) thereof, preferably comprises heavy chain constant region derived from human IgG2 or IgG4, or IgG4 without ADCC (antibody-dependent cell-mediated cytotoxicity) due to amino acid mutation.

**[0088]** The term "humanized antibody", also known as CDR-grafted antibody, refers to an antibody generated by non-human (such as murine) CDR sequences grafted onto human antibody variable region framework, i.e. antibody generated from different types of sequences of human germline antibody framework. Humanized antibody overcomes the strong anti-antibody response induced by chimeric antibody which carries a large amount of non-human (such as murine) components. Such framework sequences can be obtained from public DNA database or published references covering germline antibody gene sequences. For example, germline DNA sequences of human heavy and light chain variable region genes can be found in "VBase" human germline sequence database (available on web www.mrc-cpe.com.ac.uk/vbase), as well as found in Kabat, EA et al. 1991, Sequences of Proteins of Immunological Interest, the 5th Ed. To avoid the decrease of activity caused by reduced immunogenicity, the variable region framework of the human antibody is subjected to minimum back-mutation to maintain the activity. The humanized antibody of the present disclosure also refers to a humanized antibody which is further obtained by phage display for the purpose of CDR affinity maturation.

**[0089]** As used in the present disclosure, the term "ADCC", namely antibody-dependent cell-mediated cytotoxicity, refers to the cells expressing Fc receptors that directly kill the target cells coated by an antibody by recognizing the Fc segment of the antibody. ADCC effector function of the antibody can be reduced or eliminated by modifying the Fc segment of IgG. The modification refers to mutations on the antibody heavy chain constant region, such as mutations selected from the group consisting of N297A, L234A, L235Ain IgG1; IgG2/4 chimera; or F234A/L235A mutations in IgG4.

**[0090]** As used in the present disclosure, "identity" indicates the degree of similarity between sequences of two polynucleotides or two polypeptides. The sequence identity in the present disclosure is at least 85%, 90% or 95%, preferably at least 95%. Non-limiting examples include, but not limited to 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%. The comparison and determination of percent identity between two sequences can be accomplished using the default settings of the BLASTN/BLASTP algorithm available from the website of National Center For Biotechnology Institute.

**[0091]** The term "TGF-β receptor II" or "TGFβRII" or "transforming growth factor β receptor II" refers to binding ligands (including but not limited to TGFβ1, TGFβ2 and TGFβ3), through which the cell surface receptors trigger intracellular signaling transduction pathway.

**[0092]** The term "PD-L1" refers to programmed death ligand 1, also known as CD274 and B7H1. PD-L1 is a protein of 290 amino acids, having an extracellular IgV-like and IgC-like domain (amino acids 19-239 of full-length PD-L1), a transmembrane domain, and an intracellular domain of about 30 amino acids. PD-L1 is constitutively expressed on many cells such as antigen presenting cells (such as, dendritic cells, macrophages, and B cells), as well as hematopoietic and non-hematopoietic cells (such as, vascular endothelial cells, pancreatic islets, and immunologically privileged site). PD-L1 is also expressed on a variety of tumors and virus-infected cells, and is a member in the immunosuppressive milieu (Ribas 2012, NEJM 366: 2517-2519). PD-L1 binds to one of two T cell co-inhibitors (PD-1 and B7-1).

**[0093]** The "PD-L1 antibody or antigen-binding protein thereof" of the present disclosure include any anti-PD-L1 antibodies or antigen-binding fragments thereof described in the art. The anti-PD-L1 antibody may be a PD-L1 antibody commercially available or has been disclosed in the literatures; including but not limited to BMS-936559, MPDL3280A, MEDI4736, MSB0010718C (see US2014341917, US20130034559, US8779108) and the like. The antibody may be a monoclonal antibody, a chimeric antibody, a humanized antibody, or a human antibody. The antibody fragment includes Fab fragment, Fab' fragment, $F(ab')_2$ fragment having antigen-binding activity, and Fv fragment and scFv fragment which binds to antigen.

**[0094]** As an exemplary preparation process for PD-L1 antibody of the present disclosure, it has been published in PCT application PCT/CN2016/104320 (publication No. WO2017084495), the PD-L1 antibody comprises sequences of CDRs in heavy chain variable regions as described below:

| | | |
|---|---|---|
| HCDR1: | SYWMH | SEQ ID NO: 1 |
| HCDR2: | $RIX_1PNSGX_2TSYNEKFKN$ | SEQ ID NO: 2 |
| HCDR3: | GGSSYDYFDY | SEQ ID NO: 3. |

**[0095]** In an alternative embodiment, $X_1$ is selected from H or G; and $X_2$ is selected from G or F.

**[0096]** In another embodiment, an exemplary PD-L1 antibody of the present disclosure further comprises CDRs sequences of a light chain variable region as described below:

| | | |
|---|---|---|
| LCDR1: | RASESVSIHGTHLMH | SEQ ID NO: 4 |
| LCDR2: | AASNLES | SEQ ID NO: 5 |
| LCDR3 : | QQSFEDPLT | SEQ ID NO: 6. |

**[0097]** In another embodiment, the above CDR regions are humanized by CDR grafting strategy, and the FR of humanized light chain templates are IGKV7-3*01 and hjk2.1, the FR of humanized heavy chain templates are IGHV1-46*01 and hjh6.1, and the humanized variable region sequences are as follows:
The heavy chain variable region of humanized PD-L1 antibody:

*QVQLVQSGAEVKKPGASVKVSCKASGYTFT*SYWMH*WVRQAPGQGLEWMG*RIX$_1$P
NSGX$_2$TSYNEKFKN*RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR*GGSSYDYFDY
*WGQGTTVTVSS*;

SEQ ID NO: 7, wherein $X_1$ is selected from H or G; and $X_2$ is selected from G or F.

**[0098]** The light chain variable region of humanized PD-L1 antibody:

*DIVLTQSPASLAVSPGQRATITC*RASESVSIHGTHLMH*WYQQKPGQPPKLLIY*AASN
LES*GVPARFSGSGSGTDFTLTINPVEANDTANYYC*QQSFEDPLT*FGQGTKLEIK*
SEQ ID NO: 8;

**[0099]** NOTE: The order is FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4, italic portion represents FR sequence, and the underlined portion represents CDR sequence (the amino acid residues of CDRs are determined and denoted based on Kabat numbering criteria).

**[0100]** In another embodiment, the design for back-mutation(s) on humanized antibody of the present disclosure is performed, and the designed back mutations are shown in Table 1 below:

Table 1. back-mutation design

| VL | | VH | |
|---|---|---|---|
| VL.1 | grafted | VH.1 | grafted |
| VL.1A | Y91F | VH.1A | T74K |
| VL.1B | Y91F, G72E | VH.1B | T74K, R72V, M48I, M70L |
| VL.1C | Y91F, G72E, T22S | VH.1C | T74K, R72V, M48I, M70L, R38Q |
| | | VH.1D | T74K, R72V, M48I, M70L, R38Q, L83F |
| | | VH.1E | T74K, R72V, M48I, M70L, R38Q, L83F, V68A, V79A |
| Note: For example, Y91F indicates a back-mutation from Y to F at position 91 according to natural numbering. "Grafted" indicates that the murine antibody CDR is implanted onto human germline FR sequences. | | | |

**[0101]** New humanized antibodies can be obtained by various mutation combinations of heavy chain and light chain shown in Table 1.

**[0102]** In another aspect of the disclosure, an embodiment for constructing a humanized clone is provided, as follows: Primers were designed, and VH/VK gene fragments of each humanized antibody were constructed by PCR, and then inserted into expression vector pHr (having signal peptide and constant region gene (CH1-Fc/CL) fragment) to perform homologous recombination, in order to construct a full-length antibody expression vector: VH-CH1-Fc-pHr/VK-CL-pHr.

1. Primer Design:
The online software DNAWorks (v3.2.2) (http://helixweb.nih.gov/dnaworks/) was used to design multiple primers for synthesis of VH/VK comprising gene fragments required for recombination: 5'-30bp signal peptide + VH/VK + 30bp CH1/CL-3'.

2. Fragment splicing:
According to manuals for Primer STAR GXL DNA polymerase from TaKaRa, using the primers designed above, VH/VK comprising gene fragments required for recombination was obtained by two-step PCR amplification.

3. Construction and enzymatic digestion of expression vector pHr (having signal peptide and constant region gene (CH1-FC/CL) fragment):
The expression vector pHr (having signal peptide and constant region gene (CH1-FC/CL) fragment) was designed

and constructed by using some special restriction endonuclease, such as BsmBI which recognizes the distinctive feature between the sequence and restriction site. The vector was digested using BsmBI, and then the digested fragments were extracted by using gel and stored for use.

4. Recombinant construction of expression vector VH-CH1-Fc-pHr/VK-CL-pHr
VH/VK comprising gene fragments required for recombination and expression vector pHr (having signal peptide and constant region gene (CH1-Fc/CL) fragment) that has been digested with BsmBI were added into DH5H competent cells at a ratio of 3:1, incubated at 0°C on ice for 30min, heat-shocked at 42°C for 90s, 5 volumes of LB medium was added, and then incubated at 37°C for 45min, then plated onto LB-Amp plate, cultured at 37°C overnight. Single clone was picked for sequencing and a clone of interest was obtained.

5. The plasmid was constructed according to the design in the present example, then the purified protein was expressed, and the affinity of the obtained protein was measured by the detection described in SPR Example.

6. Finally, the affinity of the humanized back-mutation mutant(s) or hybridoma antibodies to human PD-L1-his was measured by BIACORE, the humanized back-mutation sites and combinations of sequences obtained from screening are as follows:

The heavy chain variable region of PD-L1 antibody:

*QVQLVQSGAEVKKPGASVKVSCKASGYTFT*SYWMH*WVRQAPGQGLEWMG*RIGPN SGFTSYNEKFKN*RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR*GGSSYDYFDY*WG QGTTVTVSS* SEQ ID NO: 9;

wherein HCDR2 is as shown in RIGPNSGFTSYNEKFKN SEQ ID NO: 10, i.e., $X_1$ in SEQ ID NO: 7 is G, and $X_2$ in SEQ ID NO: 7 is F;

The light chain variable region of PD-L1 antibody:

*DIVLTQSPASLAVSPGQRATITC*RASESVSIHGTHLMH*WYQQKPGQPPKLLIY*AASN LES*GVPARFSGSGSGTDFTLTINPVEAEDTANYYC*QQSFEDPLT*FGQGTKLEIK* SEQ ID NO: 11;

NOTE: The order is FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4, italic portion represents FR sequence, and the underlined portion represents CDR sequence (the amino acid residues of CDRs are determined and denoted based on Kabat numbering criteria).

**[0103]** In another aspect of the present disclosure, an embodiment for constructing and expressing an anti-PD-L1 human IgG4 type antibody is provided, and further provided is a PD-L1 antibody used for construction of fusion protein. The PD-L1 antibody can also be used as a control molecule in the Test Examples of the present disclosure.

**[0104]** Since PD-L1 is also expressed in activated T cells, therefore the use of wild-type IgG1 constant regions can cause Fc-mediated effects (such as ADCC and CDC), which could result in the reduction of activated T cells. The present disclosure selected mutated IgG4 to obtain antibodies without ADCC and CDC. The clone obtained by affinity maturation was converted into IgG4 type, and the core hinge region of IgG4 comprises S228P mutation (corresponding to the position 227 in the natural sequence of SEQ ID NO: 12). F234A (corresponding to the position 233 in the natural sequence of SEQ ID NO: 12) and L235A mutation (corresponding to the position 234 in the natural sequence of SEQ ID NO: 12) were further introduced (mAbs 4:3, 310-318; May/June 2012). At the same time, in order to avoid breakage occurred at the C-terminus of the antibody heavy chain when the linker peptide (which is used to link the TGF-βRII extracellular domain) was introduced, K on the end position of the PD-L1 antibody heavy chain was further mutated to A (corresponding to the last position in the natural sequence of SEQ ID NO: 12), so as to increase the stability of the fusion protein. The PD-L1 antibody sequence of the present disclosure used for fusion protein construction is as follows:

PD-L1 antibody heavy chain: IgG4 (AA) (S228P)

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMHWVRQAPGQGLEWMGRI
GPNSGFTSYNEKFKNRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGGSSYD
YFDYWGQGTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTV

SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTK
VDKRVESKYGPPCP*P*CPAPE*AA*GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ
EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH
EALHNHYTQKSLSLSLG*A* SEQ ID NO: 12;

NOTE: The underlined portion is the heavy chain variable region sequence, and the un-underlined portion is the heavy chain constant region sequence (the portion in italics is the mutation site);

PD-L1 antibody light chain:

DIVLTQSPASLAVSPGQRATITCRASESVSIHGTHLMHWYQQKPGQPPKLLIYAA
SNLESGVPARFSGSGSGTDFTLTINPVEAEDTANYYCQQSFEDPLTFGQGTKLEIK
RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
SEQ ID NO: 13;

NOTE: The underlined portion is the light chain variable region sequence, and the un-underlined portion is the light chain constant region sequence.

**[0105]** As used in the present disclosure, a fusion protein described in the present disclosure is a protein product obtained by co-expressing two genes via DNA recombination technology. Methods for producing and purifying antibodies and antigen-binding fragments are well known in the art and can be found (e.g., in Antibodies, A Laboratory Manual, Cold Spring Harbor, chapters 5-8 and 15). For example, mice can be immunized with human PD-L1 or fragments thereof, and the resulting antibodies can then be re-natured, purified, and sequenced for amino acid sequences by using conventional methods well known in the art. Antigen-binding fragments can also be prepared by conventional methods. The antibody or antigen binding fragments of the present disclosure are engineered to graft CDRs derived from non-human antibody into one or more human FRs. By aligning against the database of IMGT human antibody variable region germline using MOE software, human framework germline sequences can be obtained from ImMunoGeneTics (IMGT) website http://imgt.cines.fr, or from The Immunoglobulin Facts Book, 2001, ISBN 012441351.

**[0106]** The engineered antibodies or antigen binding fragments of the present disclosure may be prepared and purified using known methods. For example, cDNA sequences encoding a heavy chain and a light chain may be cloned and engineered into a GS expression vector. The engineered immunoglobulin expression vector may then be stably transfected in CHO cells. As a more recommended method known in the art, mammalian expression system will result in glycosylation of antibody, typically at highly conserved N-terminus sites in the Fc region. Stable clones may be obtained by expression of an antibody specifically binding to human PD-L1. Positive clones may be expanded in serum-free culture medium for antibody production in bioreactors. Culture medium, into which the antibody has been secreted, may be purified by conventional techniques. For example, the medium may be loaded onto a Protein A or G Sepharose FF column that has been equilibrated with a compatible buffer. The column is washed to remove nonspecific binding components. The bound antibody is eluted by pH gradient and antibody fractions are detected by SDS-PAGE, and then collected. The antibody may be filtered and concentrated using common techniques. Soluble aggregate and multimers may be effectively removed by common techniques, including size exclusion or ion exchange. The product may be immediately frozen, for example at -70°C, or may be lyophilized.

**[0107]** The "immuno-modulatory molecule" of the present disclosure can be used to attenuate the immune tolerance of cancer cells. The present disclosure uses a truncated form of the TGF-βRII extracellular domain as the immuno-modulatory molecule in the fusion protein. "TGF-β receptor II (TGF-βRII)" binds to ligands TGF-β1 and TGF-β3 with high affinity. The TGF-β RII/TGF-β complex recruits TGF-β RI to form a signal transduction complex (Won et al, Cancer Res. 1999; 59: 1273-7). The TGF-βRII extracellular domain is a 136 amino acid residue peptide from the N-terminus of TGF-βRII extracellular, an exemplary example of which is shown in SEQ ID NO: 14. Other variants of about 136 amino acids in length and derived from human TGF-βRII extracellular domain, which capable of binding to TGF-β1 and TGF-β3, also

belong to the TGF-βRII extracellular domain of the disclosure. The present disclosure has found that the structure and function of the N-terminus consecutive truncated form of the TGF-βRII extracellular domain is more stable than that of the un-truncated molecule. A fusion protein comprising the N-terminus un-truncated form of TGF-βRII extracellular domain (a polypeptide shown as aa.1-136 of SEQ ID NO: 14) is susceptible to be broken. In particular, the TGF-β RII extracellular domain which is truncated by less than 26 consecutive amino acids from N terminus is more stable; preferably, the TGF-β RII extracellular domain which is truncated by 14-26, and more preferably, truncated by 14-21 consecutive amino acids from N terminus, has a higher expression level; and most preferably, truncated by 19 or 21 consecutive amino acids.

**[0108]** The term "TGF-β receptor fusion protein" is a fusion protein comprising TGF-β receptor. In some embodiments, the TGF-β receptor fusion protein of the present disclosure is the TGF-β receptor fusion protein described in the international patent application PCT/CN2018/086451 (WO 2018205985A1). The full content of WO 2018205985A1 is incorporated entirely into the present disclosure. In some embodiments, the TGF-β receptor fusion protein is a PD-L1 antibody/TGF-βRII extracellular domain fusion protein (PD-L1/TGF-β trap), with the TGF-βRII extracellular domain served as the immuno-modulatory molecule part of the fusion protein, the PD-L1 antibody is served as the targeting part of the fusion protein, the TGF-βRII extracellular domain (for example, shown as SEQ ID NO: 14, 15, 16 or 17) is connected to the C-terminus (also known as carboxyl end) of the heavy chain of the PD-L1 antibody by a linker sequence (for example $(G_4S)_xG$, x is 3-6), to form a fusion sequence, and the fusion sequence is connected with the light chain of the PD-L1 antibody through inter-chain disulfide bond(s) to form PD-L1/TGF-β trap fusion protein finally, the structure is shown in Figure 1. In some embodiments, the TGF-β receptor fusion protein is the fusion protein described in Table 2 of Example 1 of the disclosure.

**[0109]** The term "linker" or "linker sequence" refers to a connecting peptide sequence used to connect protein domains, usually with a certain degree of flexibility, and the use of linkers will not lead to the loss of original function of the protein domain. In some embodiments of the present disclosure, the linker sequence is $(G_4S)_xG$, wherein x is 3-6, for example, the linker sequence is a polypeptide such as: $(G_4S)_3G$, $(G_4S)_4G$, $(G_4S)_5G$, or $(G_4S)_6G$.

**[0110]** "Conservative modification" or "conservative replacement or substitution" refers to substitutions of amino acids in a protein with other amino acids having similar characteristics (e.g. charge, side-chain size, hydrophobicity/hydrophilicity, backbone conformation and rigidity, etc.), such that the changes can frequently be made without altering the biological activity of the protein. Those of skilled in the art recognize that, in general, single amino acid substitution in a non-essential region of a polypeptide does not substantially alter biological activity (see, e.g., Watson et al. (1987) Molecular Biology of the Gene, The Benjamin/Cummings Pub. Co., p224 (4th edition)). In addition, substitutions of structurally or functionally similar amino acids are less likely to disrupt biological activity.

**[0111]** "Optional" or "optionally" means that the event or situation that follows may occur, but not necessarily, and the description includes the instances in which the event or circumstance does or does not occur. For example, "optionally comprising 1-3 antibody heavy chain variable region(s)" means the antibody heavy chain variable region with specific sequence can be present, but not necessarily.

**[0112]** "Administration", "administrating" and "treatment," as they apply to an animal, human, experimental subject, cell, tissue, organ, or biological fluid, refer to the contact of an exogenous pharmaceutical, therapeutic, diagnostic agent or composition to the animal, human, subject, cell, tissue, organ or biological fluid. "Administration", "administrating" and "treatment" can refer to, e.g., therapeutic, pharmacokinetic, diagnostic, research and experimental methods. Treatment of a cell encompasses contacting a reagent to a cell, as well as contacting a reagent to a fluid, where the fluid is in contact with the cell. "Administration", "administrating" and "treatment" also mean *in vitro* and *ex vivo* treatments, e.g., of a cell, by a reagent, diagnostic, binding composition, or by another cell. "Administration" or "treatment" as it applies to a human, veterinary or research subject, refers to therapeutic treatment, prophylactic or preventative measures, to research and diagnostic applications.

**[0113]** "Treat" means to administer a therapeutic agent, such as a composition of the present disclosure, internally or externally, to a subject having one or more disease symptoms for which the agent has known therapeutic activity. Typically, the agent is administered in an amount effective to alleviate one or more disease symptoms in the subject or population to be treated, to induce the regression of or prevent the progression of such symptom(s) at a clinically measurable degree. The amount of a therapeutic agent that is effective to alleviate any particular disease symptom (also referred to as the "therapeutically effective amount") may vary according to factors such as the disease state, age, and weight of the subject, and the ability of the agent to elicit a desired response in the subject. Whether a disease symptom has been alleviated can be assessed by any clinical measurement typically used by physicians or other skilled healthcare providers to assess the severity or progression status of the symptom. Although an embodiment of the present disclosure (e.g., a treatment method or article of manufacture) may not be effective in alleviating the target disease symptom(s) in every subject, it should alleviate the target disease symptom(s) in a statistically significant number of subjects as determined by any statistical test known in the art, such as the Student's t-test, the chi-square test, the U-test according to Mann and Whitney, the Kruskal-Wallis test (H-test), Jonckheere-Terpstra-test and the Wilcoxon-test.

**[0114]** "Effective amount" encompasses an amount sufficient to ameliorate or prevent a symptom or sign of the medical

condition. Effective amount also means an amount sufficient to allow or facilitate diagnosis. An effective amount for a particular subject or veterinary subject may vary depending on factors, such as the condition being treated, the overall health condition of the subject, the route and dosage of administration and the severity of side effects. An effective amount can be the maximal dosage or dosing protocol that avoids significant side effects or toxic effects.

**[0115]** "Tm value" refers to a temperature at which the thermal denaturation occurs to a protein, that is, the temperature at which half of the protein is unfolded. At this time, the spatial structure of the protein is destroyed. Therefore, the higher the Tm value, the higher the thermal stability of the protein.

**[0116]** "Substitution" refers to a replacement of the solvent system that dissolves the antibody protein. For example, the high salt or hypertonic solvent system comprising the antibody protein is replaced using physical operation against a buffer system for stable preparation, so that the antibody protein can be present in the stable preparation. The physical operation includes but not limited to ultrafiltration, dialysis or reconstitution following centrifugation.

Detailed description of the invention

**[0117]** Hereinafter, the present disclosure is further described with reference to examples, test examples or preparation examples. However, the examples, test examples or preparation examples are only for illustrative purpose, the scope of the present disclosure is not limited thereto.

**[0118]** In the examples, test examples or preparation examples of the present disclosure, where specific conditions are not described, they are generally conducted under conventional conditions or under conditions proposed by the material or product manufacturers. Where the source of the reagents is not specifically indicated, the reagents are commercially available conventional reagents.

**EXAMPLES**

**Example 1: Cloning and expression of fusion protein PD-L1/TGF-β trap**

**[0119]** The TGF-βRII extracellular domain (full length or truncated form of SEQ ID NO: 14) was used as the portion for immuno-modulatory molecule in the fusion protein, and the PD-L1 antibody is used as a targeting portion of the fusion protein to form a PD-L1 antibody/ TGF-βRII extracellular domain fusion protein (PD-L1/TGF-β trap).

**[0120]** It was surprisingly found that the truncated form of the TGF-βRII extracellular domain is relatively stable, especially more stable after being truncated by less than 26 amino acids from its N-terminus, preferably, higher expression level and more stable structure are obtained after being truncated by 14-26 amino acids, more preferably being truncated by 14-21 consecutive amino acids from N-terminus, and more preferably being truncated by 14, 19 or 21 consecutive amino acids from N-terminus.

**[0121]** The sequences of the non-limiting examples of TGF-βRII extracellular domain and its truncated form in the present disclosure are as follows:

Sequence of TGF-βRII extracellular domain: ECD (1-136)

IPPHVQKSVNNDMIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICE
KPQEVCVAVWRKNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGET

FFMCSCSSDECNDNIIFSEEYNTSNPD
SEQ ID NO: 14;

TGF-β RII extracellular domain sequence, with a truncation or deletion of 19 amino acids at the N-terminus: ECD (20-136)

GAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRKNDENIT
LETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSE
EYNTSNPD
SEQ ID NO: 15;

TGF-β RII extracellular domain sequence, with a truncation or deletion of 21 amino acids at the N-terminus: ECD (22-136)

VKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRKNDENITLET
VCHDPKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSEEYN
TSNPD
SEQ ID NO: 16;

TGF-β RII extracellular domain sequence, with a truncation or deletion of 14 amino acids at the N-terminus: ECD (15-136)

VTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRKN
DENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSCSSDECND
NIIFSEEYNTSNPD
SEQ ID NO: 17.

**[0122]** As an example, the heavy chain C-terminus amino acid of the PD-L1 antibody of the present disclosure (a PD-L1 antibody, wherein the heavy chain shown as SEQ ID NO: 12, and light chain shown as SEQ ID NO: 13) was ligated to the TGF-βRII extracellular domain with varying lengths by linker $(G_4S)_xG$ (x is 3-6), by homologous recombination technique, and was conventionally expressed in 293 expression system together with the light chain of PD-L1 antibody, and the obtained fusion proteins are shown in Table 2:

Table 2. Fusion protein of PD-L1 antibody/TGF-βRII extracellular domain

| Fusion protein | Sequence description | the number of consecutive amino acid deleted at N-terminus |
|---|---|---|
| Fusion protein 1 | Ab-$(G_4S)_4$G-ECD (1-136) | Without deletion |
| Fusion protein 2 | Ab-$(G_4S)_3$G-ECD (15-136) | 14 |
| Fusion protein 3 | Ab-$(G_4S)_3$G-ECD (15-136, N19A) | 14 |
| Fusion protein 4 | Ab-$(G_4S)_3$G-ECD (20-136) | 19 |
| Fusion protein 5 | Ab-$(G_4S)_3$G-ECD (22-136) | 21 |
| Fusion protein 6 | Ab-$(G_4S)_3$G-ECD (27-136) | 26 |
| Fusion protein 7 | Ab-$(G_4S)_4$G-ECD (15-136) | 14 |
| Fusion protein 8 | Ab-$(G_4S)_4$G-ECD (15-136, N19A) | 14 |
| Fusion protein 9 | Ab-$(G_4S)_4$G-ECD (20-136) | 19 |
| Fusion protein 10 | Ab-$(G_4S)_4$G-ECD (22-136) | 21 |
| Fusion protein 11 | Ab-$(G_4S)_4$G-ECD (27-136) | 26 |
| Fusion protein 12 | Ab-$(G_4S)_5$G-ECD (15-136) | 14 |
| Fusion protein 13 | Ab-$(G_4S)_5$G-ECD (15-136, N19A) | 14 |
| Fusion protein 14 | Ab-$(G_4S)_5$G-ECD (20-136) | 19 |
| Fusion protein 15 | Ab-$(G_4S)_5$G-ECD (22-136) | 21 |
| Fusion protein 16 | Ab-$(G_4S)_5$G-ECD (27-136) | 26 |
| Fusion protein 17 | Ab-$(G_4S)_6$G-ECD (27-136) | 26 |
| Note: Ab represents PD-L1 antibody of the present disclosure (the heavy chain shown as SEQ ID NO: 12, and light chain shown as SEQ ID NO: 13); ECD (n-136) in Sequence Description represents the full-length or truncated form of the TGF-βRII extracellular domain; n represents the starting number of amino acid after truncation of the TGF-βRII extracellular domain. The structure of the fusion protein of the present disclosure is shown in Figure 1; N19A indicates that the amino acid at position 19 of the full-length TGF-βRII extracellular domain (SEQ ID NO: 14) is mutated from N to A. | | |

**[0123]** The nucleotide sequence encoding the PD-L1 antibody, the nucleotide sequence encoding the TGF-βRII extracellular domain, and the nucleotide sequence of the linker protein fragment ($(G_4S)_xG$) were obtained by conventional technique in the art. The C-terminus nucleotide of the PD-L1 antibody was ligated through linker protein to the N-terminus

nucleotide of the TGF-βRII extracellular domain with different length by homologous recombination technique, and then cloned into the Phr-BsmbI vector. Recombinant PD-L1/TGF-β trap was expressed in 293 cells and purified as described in Example 2. The purified protein can be used in the experiments of the following examples.

**Example 2: Purification of PD-L1/TGF-β trap fusion protein**

**[0124]** The cell culture medium was centrifuged at high speed, and the supernatant was collected, and the first step of purification was performed by affinity chromatography. The chromatographic medium is Protein A or derived filler that interacts with Fc, such as GE's Mabselect. The equilibration buffer was 1×PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L $Na_2HPO_4$, 2 mmol/L $KH_2PO_4$, pH7.4). After equilibrating 5x column volumes, the cell supernatant was loaded for binding, and the flow rate was controlled so that the sample was allowed to be remained on the column for ≥ 1 min. After sample was loaded, the column was washed with 1×PBS (pH 7.4) until the A280 UV absorption was reduced to baseline. Then, the column was washed with 0.1 M glycine (pH 3.0) elution buffer, and the eluted peak was collected according to the A280 UV absorption peak, and the collected eluted sample was neutralized with 1 M Tris (pH 8.5).

**[0125]** The neutralized eluted sample was concentrated by ultrafiltration, and then subjected to size exclusion chromatography, the buffer was 1×PBS, and the column was XK26/60 Superdex 200 (GE). The flow rate was controlled at 4 ml/min, the loading volume was less than 5 ml, and the target protein peak was pooled according to A280 UV absorption. The purity of the collected protein was greater than 95% as identified by SEC-HPLC, and was verified by LC-MS. The verified sample was aliquoted for use. The PD-L1/TGF-β trap was obtained.

**[0126]** The performance and beneficial effect of PD-L1/TGF-β trap fusion protein in the present disclosure are verified by biochemical test methods as indicated below.

Test Example (Biological evaluation ***in vivo, in vitro***)

**Test Example 1: *In vitro* ELISA detection of PD-L1/TGF-β trap binding to TGF-β1**

**[0127]** The detection process is described as follows:

a. 96-well plates were coated with 100 μl/well of human TGF-β1 (8915LC, CST) at a concentration of 1 μg/ml at 4 °C overnight.

b. Washing 3 times with 250 μl of 1×PBST, 250 μl of 5% milk PBS was added for blocking at 37 °C for 2 hours.

c. Washing 3 times with 250μl of 1×PBST, gradient dilutions of PD-L1ATGF-β trap were added, and TGF-β trap was used as positive control and incubated for 1 hour at 37 °C.

d. Washing 3 times with 250 μl 1×PBST.

e. 100 μl of Anti-human Fc antibody-HRP (1:4000) was added to each well and incubated for 40 minutes at 37 °C.

f. 100 μl of TMB was added into each well, incubated for 10 minutes at room temperature, and the reaction was stopped by adding 100 μl of 1 M $H_2SO_4$.

g. The absorbance at 450 nm was measured on a microplate reader, and the data was analyzed by Graphpad Prism 5.

**[0128]** The results of binding of the fusion proteins to human TGF-β1 *in vitro* are shown in Figures 2 and 3. The ELISA showed that fusion protein 1 in Table 2 did not retain the binding activity to human TGF-β1. Mass spectrometry analysis showed that fusion protein 1 (i.e., the un-truncated form of TGF-βRII extracellular domain (1-136)) was unstable, and it was easily broken in the heavy chain TGF-βRII, and positive control has the same defect. The fusion proteins comprising the N-terminus truncated form of the extracellular domain of TGFβRII, such as fusion proteins 7, 9, 10, 12-15, specifically bind to human TGF-β1.

**Test Example 2: *In vitro* ELISA detection of PD-L1/TGF-β trap binding to PD-L1**

**[0129]** Antigen used for detection: PD-L1-His

FTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMEDKNIIQFVHGEE
DLKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQDAGVYRCMISYGGADYK
RITVKVNAPYNKINQRILVVDPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGK
TTTTNSKREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENHTAELVIPELPLAHPP
NEREQKLISEEDLHHHHHH

SEQ ID NO: 18.

**[0130]** The detection process is described as follows:

a. 96-well plates were coated with 100 μl /well of human PD-L1-His (SEQ ID NO: 18) at a concentration of 5 μg/ml at 4 °C overnight.

b. Washing 3 times with 250 μl of 1×PBST, 250 μl of 5% milk PBS was added for blocking at 37 °C for 2 hours.

c. Washing 3 times with 250μl of 1×PBST, gradient dilutions of PD-L1/TGF-β trap, and PD-L1 antibody as positive control were added, and incubated for 1 hour at 37 °C.

d. Washing 3 times with 250μl 1×PBST.

e. 100μl of Anti-human Fc antibody-HRP (1:4000) was added into each well and incubated for 40 minutes at 37 °C.

f. 100μl of TMB was added into each well, incubated for 10 minutes at room temperature, and the reaction was stopped by adding 100 μl of 1 M $H_2SO_4$.

g. The absorbance at 450 nm was measured on a microplate reader, and the data was analyzed by Graphpad Prism 5.

**[0131]** The results of binding of the fusion proteins of the present disclosure to human PD-L1 in vitro are shown in FIG.4. The ELISA showed that all fusion proteins retained the binding activity to human PD-L1.

**Test Example 3: Blocking detection of PD-1/PD-L1 pathway *in vitro***

1. Test purpose:

**[0132]** In order to investigate the blocking effect of PD-L1/TGF-β trap on PD-1/PD-L1 signaling pathway, cell-based antibody blocking experiment was performed on cells carrying human PD-1 and PD-L1 receptor molecules which were constructed by Promaga, respectively.

2. Test samples

**[0133]** ① PD-L1 antibody with heavy chain shown as SEQ ID NO: 12, and light chain shown as SEQ ID NO: 13;
**[0134]** ② Control 1 (20T-Fc): ECD(20-136)-Fc, a fusion protein comprising truncated TGF-βRII extracellular domain fragment ECD (20-136) and Fc, and the sequence is as follows:

GAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRKNDENIT
LETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSE
EYNTSNPDAESKYGPPCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVV
DVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNG
KEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFS
CSVMHEALHNHYTQKSLSLSLG SEQ ID NO: 19;

**[0135]** ③ Control 2 (22T-Fc): ECD(22-136)-Fc, a fusion protein of truncated TGF-βRII extracellular domain fragment ECD (22-136) and Fc, and the sequence is as follows:

VKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRKNDENITLET
VCHDPKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSEEYN
TSNPDAESKYGPPCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEY
KCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYP
SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVM

HEALHNHYTQKSLSLSLG SEQ ID NO: 20;

**[0136]** ④ TGF-β receptor fusion protein prepared in Example 1 of the present disclosure: the fusion protein 9, fusion protein 15:

**[0137]** In fusion protein 9, the fusion peptide sequence of PD-L1 antibody heavy chain-$(G_4S)_4$G-TGF-β RII ECD (20-136) is as follows:

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMHWVRQAPGQGLEWMGRI
GPNSGFTSYNEKFKNRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGGSSYD
YFDYWGQGTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTK
VDKRVESKYGPPCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ
EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH
EALHNHYTQKSLSLSLGA***GGGGSGGGGSGGGGSGGGGSG***GAVKFPQLCKFCD
VRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRKNDENITLETVCHDPKLPYH
DFILEDAASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPD
SEQ ID NO: 23;

NOTE: The regular font is the sequence of the heavy chain of the PD-L1 antibody, the italic is the linker sequence, and the underline is the sequence of the truncated fragment ECD (20-136) of the TGF-βRII extracellular region.

**[0138]** The light chain sequence of the PD-L1 antibody in fusion protein 9 is as follows:

DIVLTQSPASLAVSPGQRATITCRASESVSIHGTHLMHWYQQKPGQPPKLLIYAA
SNLESGVPARFSGSGSGTDFTLTINPVEAEDTANYYCQQSFEDPLTFGQGTKLEIK
RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
SEQ ID NO: 13;

**[0139]** The fusion peptide sequence of PD-L1 antibody heavy chain-$(G_4S)_5$ G-TGF-β RII ECD (22-136) in fusion protein 15 is as follows:

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMHWVRQAPGQGLEWMGRI
GPNSGFTSYNEKFKNRVTMTRDTSTSTVYMELSSLRSEDTAVYYCARGGSSYD
YFDYWGQGTTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTK
VDKRVESKYGPPCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ
EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMH
EALHNHYTQKSLSLSLGA***GGGGSGGGGSGGGGSGGGGSGGGGSG***VKFPQLCK
FCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWRKNDENITLETVCHDPKLP
YHDFILEDAASPKCIMKEKKKPGETFFMCSCSSDECNDNIIFSEEYNTSNPD
SEQ ID NO: 24;

NOTE: The regular font is the sequence of the heavy chain of the PD-L1 antibody, the italic is the linker sequence, and the underline is the sequence of the truncated fragment ECD (22-136) of the TGF-βRII extracellular region.

**[0140]** The light chain sequence of the PD-L1 antibody in fusion protein 15 is as follows:

DIVLTQSPASLAVSPGQRATITCRASESVSIHGTHLMHWYQQKPGQPPKLLIYAA
SNLESGVPARFSGSGSGTDFTLTINPVEAEDTANYYCQQSFEDPLTFGQGTKLEIK
RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
SEQ ID NO: 13;

**[0141]** ⑤ human IgG: blank control, human immunoglobulin obtained from mixed normal human serum by purification using a conventional affinity chromatography method such as Protein A;

**[0142]** ⑥ Positive control (FP17022): fusion protein of PD-L1 antibody 2/TGF-βRII extracellular domain;

**[0143]** The amino acid sequence of PD-L1 antibody 2 light chain in FP17022 fusion protein:

QSALTQPASVSGSPGQSITISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIYDVS
NRPSGVSNRFSGSKSGNTASLTISGLQAEDEADYYCSSYTSSSTRVFGTGTKVTV
LGQPKANPTVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADGSPVKAGV
ETTKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS
SEQ ID NO:21;

**[0144]** The fusion peptide amino acid sequence of PD-L1 antibody 2 heavy chain/TGF-βRII extracellular domain (1-136) in FP17022 fusion protein:

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYIMMWVRQAPGKGLEWVSSIYPS
GGITFYADTVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARIKLGTVTTVD
YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKR
VEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM
HEALHNHYTQKSLSLSPGAGGGGSGGGGSGGGGSGGGGSGIPPHVQKSVNND
MIVTDNNGAVKFPQLCKFCDVRFSTCDNQKSCMSNCSITSICEKPQEVCVAVWR
KNDENITLETVCHDPKLPYHDFILEDAASPKCIMKEKKKPGETFFMCSCSSDEC
NDNIIFSEEYNTSNPD SEQ ID NO:22;

3. Test process

**[0145]** CHO/PD-L1 cells (CS187108, Promega) were digested and resuspended in F-12 Nutrient Mixture (Ham) complete medium. The cell density was adjusted to $4\times10^5$/mL using complete medium according to the cell count results. The cell suspension was ransferred to the loading tank, added to the 96-well plate at 100 μL/well using a multi-channel pipette, and incubated at 37 °C, 5% $CO_2$ incubator for 20-24 h; The Jurkat/PD-1 (CS187102, Promega) cell suspension was prepared the next day, and the cells were resuspended according to the cell count results using assay medium, and the cell density was adjusted to $1.25\times10^6$/mL; The cell culture plates comprising CHO/PD-L1 cells were taken out from the incubator, 95μL of the culture solution was taken out per well using a multi-channel pipette, and the gradient-diluted fusion protein, PD-L1 antibody and positive coontrol (FP17022) were respectively added at 40 μL/well. Then the Jurkat/PD-1 cell suspension was transferred to a loading tank, added to the cell culture plate at 40 μL/well, and incubated at 37 °C , 5% $CO_2$ for 5-6 h. During the incubation with protein, the Bio-Glo™ Reagent was taken out and allowed to return to room temperature. Took out the cell culture plates and placed them at room temperature for 5-10 min. Then 40μL Bio-Glo™ Reagent was added to each well, incubated in a safety cabinet for 5-10 min, and the chemiluminescence signal value was read using a multi-function microplate reader.

4. Results

**[0146]** As shown in Fig. 5, similarly to positive control molecule, the fusion protein 9 of the present disclosure was able to effectively block the binding of PD-1-expressing Jurkat cells to CHO/PD-L1 cells, and there was a drug concentration and dose-dependent effect. Fusion protein 15 has the same blocking ability as that of fusion protein 9.

**Test Example 4: Binding affinity and kinetics detection in vitro by Biacore**

**[0147]** The affinity of the test molecule to human or murine TGF-β1 or human PD-L1 protein was determined by Biacore T200 (GE). The experimental procedure is described as follows:
A certain amount of PD-L1/TGF-β trap was captured with Protein A chip, and then the human or murine TGF-β1 (8915LC, CST) or human PD-L1 (Sino Biological) was flowed through the surface of the chip. The reaction signal was detected in real-time using Biacore to obtain the association and dissociation curves. The biochip was then washed and regenerated with glycine-hydrochloric acid (pH 1.5, GE). The buffer solution used in the experiment was HBS-EP Buffer (GE). The experimental data were fitted to (1:1) Langmuir model using BIAevaluation version 4.1 software (GE), and the affinity values were obtained and as shown in Table 3.

Table 3: Affinity of fusion proteins of the present disclosure to TGF-β1 or human PD-L1 *in virto*

| Fusion protein* | Affinity sample | ka (1/Ms) | kd (1/s) | KD (M) |
|---|---|---|---|---|
| Fusion protein 9 | Human TGF-β1 | 1.73E7 | 7.28E-4 | 4.22E-11 |
| Fusion protein 15 | | 2.69E7 | 6.08E-4 | 2.26E-11 |
| Fusion protein 9 | murine TGF-β | 4.33E7 | 1.33E-3 | 3.07E-11 |
| Fusion protein 15 | | 3.57E7 | 1.22E-3 | 3.42E-11 |
| Fusion protein 9 | human PD-L1 | 1.97E6 | 1.24E-4 | 6.31E-11 |
| Fusion protein 15 | | 2.00E6 | 1.24E-4 | 6.10E-11 |
| * The form of fusion protein is shown in Table 2. | | | | |

**[0148]** The fusion protein binding activity is shown in Table 3. The results indicate that the fusion protein 9 and fusion protein 15 of the present disclosure have extremely high affinity to human, murine TGF-β1 and human PD-L1.

**Test Example 5: SMAD3 reporter gene inhibition assay**

**[0149]**

1. Test purpose:
In this experiment, the Smad3 binding element (SBE) with luciferase reporter gene was expressed in HepG2 cells to study the inhibitory effect of PD-L1/TGF-β trap on TGF-β1-induced Smad3 activation, and the activity of PD-L1/TGF-β trap *in virto* was evaluated according to IC50 vaule.

2. Test Sample: fusion protein 9, positive control (FP17022).

3. Test process

**[0150]** HepG2 cells were cultured in MEM complete medium (GE, SH30243.01) comprising 10% FBS and sub-cultured every 3 days. On the first day of the experiment, 25,000 cells per well were inoculated to 96-well plates (Corning, 3903), and cultured at 37 °C, 5% $CO_2$ for 24 hours. On the next day, the medium in the cell culture plates was discarded, and 100 ng of 3TP-Lux plasmid was transfected per well. The cells were further cultured at 37 °C, 5% $CO_2$ for 24 hours. Six hours before the addition of the test sample, the complete medium in the 96-well plate was discarded, and 80 μL of incomplete medium (MEM + 0.5% FBS) was added to each well. After 6 hours, 10 μL of human TGF-β1 (R&D, 240-B-010) solution prepared in incomplete medium (final concentration of 2 ng/mL) and 10 μL of the test sample (the final concentration is 500, 50, 5, 0.5, 0.05, 0.005, 0.0005 and 0 nM) were added, the human TGF-β1 solvent was used as a control, and the cells were cultured at 37 °C, 5% $CO_2$ for another 18 h. Then, 100 μL of the prepared luciferase substrate ONE-Glo™ Luciferase Assay system (promega, E6110) was added to each well, and incubated at room temperature for 10 minutes in dark, and then the luminescent signal vaule was read using a Victor 3 multi-plate reader (Perkin Elmer). The IC50 value of the test sample was obtained by calculating using the data software Graphpad Prism 5.0.

**[0151]** Figure 6 showed that fusion protein 9 inhibited TGFβ-induced pSMAD3 reporter activity in a dose-dependent manner, and had efficacy and $IC_{50}$ (concentration required to inhibit 50% of maximum activity) comparable to that of positive control FP17022. The test results of the PD-L1 antibody showed that it had no inhibitory effect ($IC_{50}$>500 nM).

**Test Example 6: *In vitro* Detection of IFNγ secretion by PBMC due to tuberculin (TB) stimulation**

1. Test purpose

**[0152]** To investigate the activation of T lymphocytes by PD-L1/TGF-β trap, human peripheral blood mononuclear cells (PBMC) were collected and purified, and were stimulated *in vitro* with tuberculin (TB) for 5 days to detect the secretion level of IFNγ cytokine.

2. Test sample

**[0153]**

① Human IgG;
② PD-L1 antibody;
③ Fusion protein 9;
④ Control 1 (20T-Fc): ECD (20-136)-Fc;
⑤ PD-L1 antibody+control 1 (20T-Fc).

3. Test process

**[0154]** 20 μL tuberculin was added into freshly isolated and purified PBMCs, 15 mL, about $3\times10^7$, and cultured in an incubator for 5 days at 37 °C, 5% $CO_2$. On day 6, the cultured cells were collected and centrifuged, washed once with PBS and resuspended in fresh medium with the density adjusted to $1 \times 10^6$ cell/ml, 90μl of resuspended cells were added into the 96-well plate. 10μL/well of different concentrations of antibodies were separately added to corresponding wells of the above 96-well cell culture plate, 10μl PBS was added in the control and blank group, respectively. Then, the cell culture plate was incubated in the incubator for three days at 37°C, 5% $CO_2$. The cell culture plate was taken out, and the supernatant was taken from each well after centrifugation (4000 rpm, 10 min). After 10-fold dilution, the secretion of IFN-γ was detected by ELISA (human IFN-γ detection kit, NEOBIOSCIENCE, EHC 102g.96), according to the reagent instructions for specific operations. As shown in Table 4, all the PD-L1/TGF-β trap fusion protein samples were able to enhance the secretion of cytokine IFN-γ by the activated T lymphocytes, and there was a drug concentration dose effect.

Table 4. The secretion result of cytokine IFN-γ

| Antibody | EC50 (nM) | Maximum secretion of IFNγ (pg/ml) | Minimal secretion of IFNγ (pg/ml) | Fold (secretion of IFNγ) |
|---|---|---|---|---|
| PD-L1 antibody | 0.05 | 2684 | 737 | 3.6 |

(continued)

| Antibody | EC50 (nM) | Maximum secretion of IFNγ (pg/ml) | Minimal secretion of IFNγ (pg/ml) | Fold (secretion of IFNγ) |
|---|---|---|---|---|
| Fusion protein 9 | 0.12 | 3422 | 638 | 5.4 |
| Control 1(20T-Fc) | >50 | 780 | 490 | 1.6 |
| PD-L1 antibody + control 1 | 0.054 | 2879 | 746 | 3.9 |
| Human IgG | >50 | 375 | 298 | 1.2 |
| Blank control | / | 536 | 536 | 1 |

4. Result

**[0155]** As shown in Figure 7 and Table 4, the fusion protein 9 was able to enhance the activated T lymphocyte to secrete cytokine IFN-γ in dose-dependent manner, and had a stronger activation effect than that of the PD-L1 antibody and 20T-FC.

**Test Example 7: Pharmacokinetic evaluation**

**[0156]** Three SD rats, female, were purchased from Jie Si Jie Laboratory Animal Co., Ltd. and maintained in 12/12-hour light-dark cycle (the temperature was 24±3°C, the relative humidity was 50-60%), the rats were free access to water and diet. On the day of the experiment, SD rats were injected with fusion protein in the tail vein at a dose of 6 mg/kg and an injection volume of 5 ml/kg.

**[0157]** Blood was collected at time point: 15 min, 7 h (on the first day), 24h (2nd day), 3rd day, 4th day, 6th day, 8th day, 10th day, and 15th day after administration, 200 μl blood (equivalent to 100 μl serum) was taken from the fundus vein of the rat. The blood sample was placed at room temperature for 30min to allow agglutination, and then centrifuged at 10000 g for 10 minutes at 4 °C. The supernatant were taken and stored at-80°C immediately. The concentration of the fusion protein in the serum was measured by ELISA.

**[0158]** The measure process is described as follows:

a. 96-well plates were coated with 100 μl/well of human PD-L1-His at a concentration of 2 μg/ml, overnight at 4 °C.
b. Washing 4 times with 250 μl of 1×PBST, 250 μl of 5% milk PBS was added for blocking at 37 °C for 3 hours.
c. Washing 4 times with 250 μl of 1×PBST, 100 μl of the gradient-diluted serum sample was added, and incubated at 37 °C for 1 hour, with fusion protein 9 served as positive control.
d. Washing 5 times with 250 μl 1×PBST.
e. 100 μl/well of biotinylated anti-human TGF-βRII antibody (R&D) was added, and incubated for 1 hour at 37 °C.
f. Washing 5 times with 250 μl 1×PBST.
g. 100 μl/well of TMB was added, incubated for 10 minutes at room temperature, and the reaction was stopped by adding 100 μl of 1 M $H_2SO_4$.
h. The absorbance at 450 nm was measured on a micro-plate reader, and the data was analyzed by Graphpad Prism 5.

Table 5: T1/2 of fusion protein in SD rat

| Test drug | Administration mode | T1/2 (Mean ± SD, h) |
|---|---|---|
| Fusion protein 9 | IV (6 mg/kg) | 236±10 |

**[0159]** The results of PK analysis indicated that the half-life of the fusion protein 9 of the present disclosure in rats was about 236 h (9.8 days), see table 5.

**Test Example 8: Effect of PD-L1/TGF-β trap on murine subcutaneous xenograft of human breast cancer MDA-MB-231**

**[0160]** The murine strain used in this experiment was a NOD/SCID female mouse (Cavens). The human peripheral blood mononuclear cells used in the experiment were extracted from freshly collected blood, and the extraction method was as follows: The heparin anti-coagulated venous blood was mixed with the same volume of PBS containing 2% FBS, and after mixing, 25 ml of the diluted blood was slowly added to a centrifuge tube containing 15 ml of lymphocyte

separation solution, and centrifuged at 1200 g for 10 minutes at room temperature. The lymphocyte layer was pipetted to another centrifuge tube; cells were washed by PBS and centrifuged at 300g for 8 minutes at room temperature. After repeated once, the cells were re-suspended in RPMI-1640 medium containing 10% FBS, and the cells were added to a 6-well plate pre-coated with CD3 antibody (OKT3, 40 ng/ml) at $2\times10^6$ cells/well (2 ml), and then placed in a 37 °C incubator for 4 days.

Test sample:

**[0161]**

① blank control: PBS;
② fusion protein 9: 4.8mpk;
③ fusion protein 9: 24mpk;
④ PD-L1 antibody: 4mpk;
⑤ PD-L1 antibody: 20mpk;
⑥ PD-L1 antibody 4mpk + control 1 (20T-Fc) 2.14mpk;
⑦ Control 1 (20T-Fc): 2.14mpk.

**[0162]** MDA-MB-231 cells were re-suspended in serum-free RPMI-1640 medium, and mixed with an equal volume of Matrigel, 100μl ($2.3\times10^6$) was inoculated subcutaneously into the right flank of NOD/SCID mice. 11 days later, animals bearing oversized or undersized tumor were excluded, mice were randomized into groups, with 9 animals in each group. $5\times10^5$ stimulated PBMCs (60 μl) were injected into the tumor tissues, and the remaining PBMCs were further cultivated without stimulation. One week later, $5\times10^6$ PBMCs (100 μl) were intraperitoneally injected into tumor-bearing mice, as the first round of injection. Throughout the experimental period, 2 and a half-round, a total of 5 PBMC injections were provided. On the day of the first intratumoral injection, intraperitoneal administration was performed, three times per week for a total of 14 administrations. The administration regimen was shown in Table 6. The tumor volume and body weight were measured twice a week. The experimental results are shown in Table 7. At the end of the experiment, the tumor-bearing mice were euthanized and the tumor was removed and weighed.

Table 6: Test grouping and administration

| Group | Administration Dose |
|---|---|
| ① Blank control: PBS | 0 |
| ② Fusion protein 9- 4.8mpk | 4.8 mg/kg |
| ③ Fusion protein 9- 24mpk | 24mg/kg |
| ④ PD-L1 antibody- 4mpk | 4mg/kg |
| ⑤ PD-L1 antibody - 20mpk | 20mg/kg |
| ⑥ PD-L1 antibody- 4mpk+control 1- 2.14mpk | 4 mg/kg +2.14 mg/kg |
| ⑦ Control 1- 2.14mpk | 2.14mg/kg |

Table 7: Effect of fusion protein 9 on murine subcutaneous xenograft of MDA-MB-231

| Group | **Day 0** | **Day 25** | | **Day 32** | | **Day 33** | |
|---|---|---|---|---|---|---|---|
| | Mean± SEM | Mean± SEM | % TGI | Mean± SEM | %TGI | Mean± SEM | P(*vs* PBS) |
| | (V $mm^3$) | (V $mm^3$) | | (V $mm^3$) | | (TW g) | (TW) |
| ① Blank control: PBS | 62.5±2.9 | 623.4± 43.3 | - | 941.1± 54.9 | - | 0.859± 0.063 | - |
| ② Fusion protein 9-4.8mpk | 62.6±3.5 | 414.6± 17.1*** | 37.24 % | 618.9± 28.7*** | 36.6 8% | 0.454± 0.025*** | 2.06E-05 |
| ③ Fusion protein 9-24mpk | 62.7±3.3 | 329.8± 22.5*** | 52.38 % | 495.3± 42.6*** | 50.7 6% | 0.367± 0.026*** | 2.20E-06 |

(continued)

| Group | Day 0 | Day 25 | | Day 32 | | Day 33 | |
|---|---|---|---|---|---|---|---|
| | Mean± SEM | Mean± SEM | % TGI | Mean± SEM | %TGI | Mean± SEM | P(*vs* PBS) |
| | (V $mm^3$) | (V $mm^3$) | | (V $mm^3$) | | (TW g) | (TW) |
| ④ PD-L1 antibody - 4mpk | 63.1±3.5 | 454.4± 40.8* | 30.24 % | 722.8± 65.8* | 24.91% | 0.592± 0.052** | 0.0050 |
| ⑤ PD-L1 antibody - 20mpk | 62.6±3.3 | 466.4± 17.2** | 28.01 % | 741.8± 32.9** | 22.70% | 0.650± 0.033** | 0.0100 |
| ⑥ PD-L1 antibody - 4mpk+control 1-2.14mpk | 62.6±3.3 | 447.5± 29.6** | 31.38 % | 669.2± 45.3** | 30.96% | 0.566± 0.039** | 0.0012 |
| ⑦ Control 1 - 2.14mpk | 60.7±3.3 | 601.5± 30.9 | 3.58 % | 861.7± 34.2 | 8.83 % | 0.652± 0.041* | 0.0178 |
| Day 0: time for the first administration; *$p$<0.05 **$p$<0.01 ***$p$<0.001, when compared with PBS by Student's t test. | | | | | | | |

**[0163]** The results are shown in Figure 8, antibody fusion protein 9 (4.8 mg/kg, 24 mg/kg) can significantly inhibit the growth of murine subcutaneous xenograft of human breast cancer MDA-MB-231. There was a dose-dependent relationship between high and low doses, and it was superior to reference drug PD-L1 antibody (4 mg/kg, 20 mg/kg), TGF-βRII control molecule 20T-FC (2.14 mg/kg) and the combination group (PD-L1 antibody -4 mg/kg + 20 T-FC - 2.14 mg/kg) at equivalent molar dose, respectively. Each dose of fusion protein 9 maintained a desired anti-tumor effect since the $14^{th}$ day after administration; when compared with PD-L1 antibody-20mpk, fusion protein 9 at high dose had obvious advantage ($p<0.05$). On the $25^{th}$ days after administration, the anti-tumor effect of each antibody reached an optimum level. The anti-tumor rate of the low and high dose of fusion protein 9 and PDL-1 antibody and the combination group was 37.24%, 52.38%, 30.24%, 28.01%, and 31.38%, respectively. On the $32^{th}$ days after administration, the antitumor effect of fusion protein 9 was still very significant. The %TGI of the low and high dose group was 36.68% and 50.76%, respectively, and the tumor volume was statistically different, when compared with the control group ($p<0.05$).

**Test Example 9: Physical stability of PD-L1/TGF-β trap**

**[0164]** This test example was used to detect the stability of fusion protein 9 and fusion protein 15.

**[0165]** DSC (Differential scanning calorimetry) was used to detect the thermal stability of different antibodies, and the stability in different buffer systems was compared. Buffer systems comprise such as lOmM acetate/135 mM NaCl (pH 5.5) and lOmM acetate/9% trehalose (pH 5.5).

**[0166]** The sample was dissolved in the corresponding buffers, and the concentration was controlled at about 50mg/ml. The detection was performed by MicroCal* VP-Capillary DSC (Malvern). Prior to test, each sample and blank buffer were degassed for 1 to 2 min using a vacuum degassing device. Each well of the plate was added with 400μl sample or blank buffer (the loading quantity was 300μl). Finally, two pairs of well-plates were added with 14% Decon 90 and $ddH_2O$, respectively, and were ready to wash. The sample was loaded on the plate, and then the plate was sealed with a plastic cover. Scanning began with a temperature at 25°C and ended at 100°C, and the scanning rate is 60°C/h. The results are shown in table 8, indicating that both fusion protein 9 and fusion protein 15 show good thermal stability in these two test systems.

Table 8. Thermal stability test

| Sample | Buffer | Tm-onset (°C) | TM (°C) |
|---|---|---|---|
| Fusion protein 9 | 10mM acetate /135mM NaCl | 57.99 | 66.33 |
| | 10mM acetate /9% trehalose | 58.64 | 67.83 |
| Fusion protein 15 | 10mM acetate /135mM NaCl | 57.33 | 66.17 |
| | 10mM acetate /9% trehalose | 57.41 | 67.44 |

**[0167]** The periodic stability at certain concentration was investigated by monitoring purity via SEC-HPLC, exemplary conditions, for example, the concentration of the sample was controlled at about 50mg/ml, in 10 mM acetate/135mM

NaCl (pH5.5), and the stability was compared under the conditions such as 5 cycles of freezing and thawing at -80°C versus after storage at 40°C for one month. Xbridge protein BEH SEC 200A (Waters) HPLC column was used for detection. The results are shown in table 9 as follows, these two fusion protein showed good stability.

Table 9. stability

| | fusion protein 9(Δ%) | fusion protein 15(Δ%) |
|---|---|---|
| 40°C | 3.39% | 1.8% |
| -80°C freeze-thaw | 1.44% | 1.39% |
| Note: Δ% indicates the rate of change. | | |

**Test Example 10: Chemical stability of fusion protein**

**[0168]** Deamidation is a common chemical modification which will influence the stability of antibody in later stage, especially it is generally chosen to avoid or to reduce the highly deamidated modification of some amino acids in the CDR regions as much as possible via mutation. 1600μg antibody to be tested was dissolved in 200 μl lOmM acetate/135mM NaCl (pH5.5), and placed in 40°C incubator. Samples were taken on day 0, 14 and 28 for enzymatic hydrolysis assay. 100μg of each sample taken at different time points was dissolved in 100μl 0.2 M His-HCl, 8 M Gua-HCl solution, pH 6.0; 3μl 0.1g/mL DTT was added, and then the sample was incubated in 50°C water bath for 1 hour. Then the sample was ultrafiltrated twice with 0.02M His-HCl (pH 6.0), and digested overnight at 37°C in water bath by adding 3μL 0.25mg/mL trypsin,. The deamidation modification was examined using an Agilent 6530 Q-TOF LC-MS, and the results are shown in Table 10 below.

Table 10. Deamidation modification

| Sample | Heavy chain | Modification site | Day 0 | Day 14 | Day 28 |
|---|---|---|---|---|---|
| Fusion protein 9 | Heavy chain | N314 | 2.38% | 2.28% | 2.45% |
| | | N324 | 0.20% | 3.60% | 7.88% |
| Fusion protein 15 | Heavy chain | N314 | 2.87% | 2.86% | 2.87% |
| | | N324 | 0.00% | 3.61% | 7.93% |
| Note: N represents the detectable modified asparagine, and the number represents the position in the light chain or heavy chain from N-terminus. The percent content represents the ratio of deamidation modification detected by LC-MS to the signal of all peptides at that site. | | | | | |

**[0169]** The results of mass spectrometry showed that the two fusion proteins don't have obvious deamidation modification sites, suggesting that the fusion proteins have good chemical stability.

**Preparation Example**

**Exemplary preparation processes for fusion protein pharmaceutical composition (preparation)**

**[0170]** The first step: a certain amount of stock solution of purified TGF-β receptor fusion protein was taken, and solvent replacement (preferably by ultrafiltration) was performed using a protein-free buffer (such as lOmM, pH 6.2 citric acid-sodium citrate buffer) by passing through an ultrafiltration membrane for at least 6-fold volume, then the protein was concentrated to about 70 mg/mL. A certain volume of sucrose stock solution was added and mixed to achieve a final sucrose concentration of 80mg/mL. A certain volume of Tween-80 stock solution was added and mixed to achieve a final Tween-80 concentration of 0.4mg/mL. lOmM pH 6.2 citrate buffer was added to reach a specified volume so as to obtain a concentration of 50mg/mL protein (other preparations to be tested or stable preparations were prepared according to similar steps).

**[0171]** After having been filtrated, the product was sampled for sterility test due to medium-control purpose. The stock solution passed through a 0.22 μm PVDF filter and the filtrate was collected.

**[0172]** The second step: the filling volume was adjusted to 6.3 ml, the filtrate was loaded into a 6ml vial, which was then capped with a stopper, and samples were taken at the beginning of, in the middle of, and at the end of filling in order to detect the difference in filling volume, due to medium-control purpose.

**[0173]** The third step: the capping machine was started, aluminum caps were capped.

**[0174]** The fourth step: visual inspection was performed to confirm that the product has no defects such as inaccurate loading. Labels were printed and labelled on vials; carton labels were printed, cartons were folded, loaded with vials, and labelled.

**Preparation Example 1. Screening of pH value for preparation buffer system of TGF-β receptor fusion protein**

**[0175]** TGF-β receptor fusion protein (fusion protein 9) preparations were prepared using the following buffers, with a protein concentration of 50 mg/ml:

1) lOmM histidine-acetic acid, pH 5.0;
2) lOmM histidine-acetic acid, pH 6.0;
3) lOmM histidine-acetic acid, pH 6.5;
4) lOmM sodium dihydrogen phosphate-disodium hydrogen phosphate, pH 7.0;
5) lOmM sodium dihydrogen phosphate-disodium hydrogen phosphate, pH 7.5.

**[0176]** Each preparation was filtrated, and added at 1.2 mL/vial into a 2 mL injection vial made of neutral borosilicate glass. The injection vial was provided with a stopper, capped and sealed. The samples were taken and subjected to a high temperature of 40°C and shaking experiments. The experimental results are shown in Table 11. The results show that TGF-β receptor fusion proteins have better stability at pH 6.0-6.5.

Table 11. Screening results of forced degradation experiment

| No. | Time point | Appearance | SEC (%) | | |
|---|---|---|---|---|---|
| | | | aggregate | monomer | fragment |
| 1 | T0 | strong opalescence | 2.0 | 97.1 | 1.0 |
| | with shaking D7 | turbid | 3.5 | 94.8 | 1.7 |
| | 40 °C M2 | clear and colorless | 8.1 | 87.1 | 4.7 |
| 2 | T0 | light blue opalescence | 2.7 | 97.0 | 0.3 |
| | with shaking D7 | turbid | 3.0 | 96.2 | 0.9 |
| | 40 °C M2 | clear and colorless | 5.9 | 91.1 | 3.0 |
| 3 | T0 | clear and colorless | 2.7 | 96.9 | 0.3 |
| | with shaking D7 | large amount of flocculent precipitate | 3.0 | 95.7 | 1.3 |
| | 40 °C M2 | clear and colorless | 5.0 | 91.7 | 3.3 |
| 4 | T0 | colorless and fine particles | 3.1 | 96.5 | 0.5 |
| | with shaking D7 | large amount of flocculent precipitate | 3.6 | 95.3 | 1.2 |
| | 40 °C M2 | clear and colorless | 4.5 | 71.5 | 23.9 |
| 5 | T0 | colorless and fine particles | 3.2 | 96.5 | 0.4 |
| | with shaking D7 | large amount of flocculent precipitate | 3.7 | 95.0 | 1.3 |
| | 40 °C M2 | clear and colorless | 4.9 | 60.8 | 34.3 |
| Note: The shaking condition was: D1: 130 rpm, D2: 200 rpm, D3-D7: 300 rpm; D means day, T means time, and M means month. | | | | | |

**Preparation Example 2. Screening of buffer system for TGF-β receptor fusion protein preparations**

**[0177]** TGF-β receptor fusion protein (fusion protein 9) preparations were prepared using the following buffers, with a protein concentration of 50 mg/ml:

1) 10 mM succinic acid-sodium succinate, pH 6.0;
2) 10 mM citric acid-sodium citrate, pH 6.0;

3) 10 mM citric acid-sodium citrate, pH 6.5;
4) lOmM sodium dihydrogen phosphate-disodium hydrogen phosphate, pH 6.5;
5) lOmM histidine-hydrochloride, pH 6.5.

**[0178]** Each preparation was filtrated, and added at 1.2 mL/vial into a 2 mL injection vial of neutral borosilicate glass. The injection vial was provided with a stopper, capped and sealed. The samples were taken for shaking (at 25°C, 300 rpm) experiment. The experimental results are shown in Table 12. The results show that a large amount of small particles were observed in the group of sodium dihydrogen phosphate-disodium hydrogen phosphate on the 6th day under shaking, and the aggregates reached 1.8% detected by SEC. However, only tiny particles were occasionally observed in other groups. It can be seen that the stability of TGF-β receptor fusion protein in citric acid, histidine and succinate buffer systems is better than that in phosphate buffer systems.

Table 12. screening experiment results for buffer system and pH value

| No. | Time point | Appearance | SEC (%) | | |
|---|---|---|---|---|---|
| | | | aggregate | monomer | fragment |
| 1 | D0 | clear and colorless | 1.6 | 98.1 | 0.3 |
| | with shaking D6 | tiny particles occasionally | 1.7 | 97.7 | 0.6 |
| 2 | D0 | clear and colorless | 1.5 | 98.0 | 0.5 |
| | with shaking D6 | tiny particles occasionally | 1.5 | 97.8 | 0.7 |
| 3 | D0 | clear and colorless | 1.6 | 98.0 | 0.4 |
| | with shaking D6 | tiny particles occasionally | 1.7 | 97.7 | 0.6 |
| 4 | D0 | clear and colorless | 1.6 | 98.0 | 0.4 |
| | with shaking D6 | large amount of tiny particles | 1.8 | 97.6 | 0.7 |
| 5 | D0 | clear and colorless | 1.5 | 98.0 | 0.5 |
| | with shaking D6 | tiny particles occasionally | 1.6 | 97.8 | 0.7 |
| Note: D represents days. | | | | | |

**Preparation Example 3. Further screening of buffer system for TGF-β receptor fusion protein preparation**

**[0179]** A buffer of pH 6.2 comprising 10 mM histidine-hydrochloride or 10 mM citric acid-sodium citrate was used to prepare a preparation comprising 80 mg/ml sucrose, 0.4 mg/ml polysorbate 80, TGF-β receptor fusion protein (fusion protein 9) at a concentration of 50 mg/ml.

**[0180]** Each preparation was filtrated, and added at 1.2 mL/vial into a 2 mL injection vial made of neutral borosilicate glass. The injection vial was provided with a stopper, capped and sealed. The samples were stored at 25°C for stability analysis, 6-month SEC or non-reducing CE-SDS detection.

**[0181]** The experimental results are shown in Table 13. The results show that the citric acid-sodium citrate system is better than the histidine-hydrochloride system (M6 SEC aggregate: 1.8% v.s. 2.2%; non-reducing CE-SDS: 94.5% v.s. 92.2%); Thus, the citric acid system can be selected as the buffer system for TGF-β receptor fusion protein.

Table 13. Accelerated stability test results for buffer system screening at 25°C

| Buffer system | Time | Appearance | SEC (%) | | | Non-reducing CE-SDS (%) |
|---|---|---|---|---|---|---|
| | | | aggregate | monomer | fragment | |
| citrate buffer system | T0 | clear | 1.6 | 97.6 | 0.7 | 91.2 |
| | D24 | clear | 1.6 | 97.7 | 0.7 | 90.4 |
| | M2 | clear | 1.7 | 97.5 | 0.8 | N/A |
| | M3 | clear | 1.8 | 97.9 | 0.3 | 96.2 |
| | M6 | large amount of cloudy particles | 1.8 | 97.9 | 0.4 | 94.5 |
| histidine salt buffer system | T0 | clear | 1.5 | 97.7 | 0.8 | 91.3 |
| | D24 | clear | 1.6 | 97.4 | 1.1 | 90.4 |
| | M2 | clear | 1.7 | 97.5 | 0.8 | N/A |
| | M3 | clear | 1.8 | 97.7 | 0.5 | 95.4 |
| | M6 | large amount of cloudy particles | 2.2 | 97.3 | 0.5 | 92.2 |
| Note: T means time; D means day; M means month. | | | | | | |

**Preparation Example 4. Screening of stabilizers for TGF-β receptor fusion protein preparations**

**[0182]** TGF-β receptor fusion protein (fusion protein 9) preparations were prepared using the following buffers of different saccharides, with a protein concentration of 50 mg/ml:

1) lOmM citric acid-sodium citrate, 80 mg/ml sucrose, pH 6.2;
2) lOmM citric acid-sodium citrate, 80 mg/ml α,α-trehalose dihydrate, pH 6.2.

**[0183]** Each preparation was filtrated, and added at 1.2 mL/vial into a 2 mL injection vial made of neutral borosilicate glass. The injection vial was provided with a stopper, capped and sealed. The samples were taken for long-term storage experiments at 25°C room temperature and at 2-8°C low temperature.

**[0184]** The experimental results are shown in Table 14. The results show that sucrose and trehalose have similar effects on the stability of TGF-β receptor fusion protein (fusion protein 9). Sucrose was selected as the stabilizer of TGF-β receptor fusion protein (fusion protein 9). When the sucrose concentration is 80 mg/ml, the osmotic pressure is about 300 mosm/kg which is close to being isotonic, therefore the sucrose concentration can be 80 mg/ml.

Table 14. Results of screening experiments for types of saccharide

| No. | Time point | Appearance | SEC (%) | | | Non-reducing CE-SDS (%) |
|---|---|---|---|---|---|---|
| | | | aggregate | monomer | fragment | |
| 1 | T0 | clear and colorless | 1.6 | 97.6 | 0.7 | 91.2 |
| | 25°C M6 | large amount of cloudy particles | 1.8 | 97.9 | 0.4 | 94.5 |
| | 2-8°C M6 | clear and colorless | 1.7 | 98.1 | 0.1 | 96.8 |
| 2 | T0 | clear | 1.6 | 97.7 | 0.7 | 91.6 |
| | 25°C M6 | significant cloudy particles | 1.9 | 97.8 | 0.3 | 94.1 |
| | 2-8°C M6 | clear and colorless | 1.8 | 97.8 | 0.4 | 97.5 |
| Note: T means time, and M means month. | | | | | | |

**Preparation Example 5. Screening of surfactants for TGF-β receptor fusion protein preparations**

**[0185]** TGF-β receptor fusion protein (fusion protein 9) preparations were prepared using the following buffers of different types surfactants at different concentrations, with a protein concentration of 50 mg/ml:

1) lOmM histidine-hydrochloride, 0.1 mg/ml polysorbate 20, pH 6.2;
2) lOmM histidine-hydrochloride, 0.2 mg/ml polysorbate 20, pH 6.2;
3) lOmM histidine-hydrochloride, 0.4 mg/ml polysorbate 20, pH 6.2;
4) lOmM histidine-hydrochloride, 0.6 mg/ml polysorbate 20, pH 6.2;
5) lOmM histidine-hydrochloride, 0.8 mg/ml polysorbate 20, pH 6.2;
6) lOmM histidine-hydrochloride, 0.1 mg/ml polysorbate 80, pH 6.2;
7) lOmM histidine-hydrochloride, 0.2 mg/ml polysorbate 80, pH 6.2;
8) lOmM histidine-hydrochloride, 0.4 mg/ml polysorbate 80, pH 6.2;
9) lOmM histidine-hydrochloride, 0.6 mg/ml polysorbate 80, pH 6.2;
10) lOmM histidine-hydrochloride, 0.8 mg/ml polysorbate 80, pH 6.2.

**[0186]** Each preparation was filtrated, 0.5 mL of preparation was injected into 50 mL saline injection or into 5% glucose injection solution, to reach a protein concentration of 0.5 mg/mL after dilution. The sample stability after dilution was observed. The results of the experiment are shown in Table 15. The results show that when the concentration of polysorbate 20 in the preparation reached more than 0.2 mg/ml, the insoluble particles decreased significantly after dilution; as for polysorbate 80, the insoluble particles produced due to sodium chloride dilution decreased along with the increase of polysorbate 80 concentration. When polysorbate 80 reached 0.4 mg/ml or more, particles larger than 10 μm was reduced to less than 10 particles/ml.

Table 15. results of polysorbate screening - dilution and shaking experiment

| No. | Insoluble particles after dilution (particles/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 0.9% NaCl | | | 5% Glucose | | |
| | 2μm | 10μm | 25μm | 2μm | 10μm | 25μm |
| 1 | 1454 | 18 | 0 | 318 | 10 | 0 |
| 2 | 48 | 1 | 0 | 104 | 2 | 0 |
| 3 | 65 | 2 | 0 | 177 | 3 | 0 |
| 4 | 26 | 1 | 0 | 102 | 1 | 0 |
| 5 | 112 | 3 | 0 | 82 | 2 | 0 |
| 6 | 568 | 36 | 1 | 46 | 1 | 0 |
| 7 | 668 | 14 | 0 | 30 | 1 | 0 |
| 8 | 135 | 3 | 0 | 92 | 4 | 0 |
| 9 | 623 | 8 | 0 | 30 | 1 | 0 |
| 10 | 113 | 2 | 0 | 97 | 6 | 0 |

**Preparation Example 6. Further screening of surfactants for TGF-β receptor fusion protein preparations**

**[0187]** TGF-β receptor fusion protein (fusion protein 9) preparations were prepared using the following buffers of different types surfactants, with a protein concentration of 50 mg/ml:

1) lOmM citric acid-sodium citrate, 0.4 mg/ml polysorbate 80, pH 6.2;
2) lOmM citric acid-sodium citrate, 0.6 mg/ml polysorbate 20, pH 6.2.

**[0188]** Each preparation was filtrated, and added at 1.2 mL/vial into a 2 mL injection vial made of neutral borosilicate glass. The injection vial was provided with a stopper, capped and sealed. The samples were taken for long-term storage experiments at 2-8°C low temperature.

**[0189]** The experimental results are shown in Table 16. The results indicate that polysorbate 80 has a better stability

effect on TGF-β receptor fusion protein (fusion protein 9). Therefore, polysorbate 80 was selected as surfactant for TGF-β receptor fusion protein (fusion protein 9).

Table 16. Results of long-term stability experiment at 2-8 °C for screening Polysorbate

| No. | Time point | Appearance | SEC (%) | | | Non-reducing CE-SD (%) |
|---|---|---|---|---|---|---|
| | | | aggregate | monomer | fragment | |
| 1 | T0 | clear and colorless | 1.6 | 97.6 | 0.7 | 91.2 |
| | D45 | clear and colorless | 1.7 | 97.4 | 1.0 | N/A |
| | M3 | clear and colorless | 1.8 | 98.0 | 0.3 | 97.4 |
| | M6 | clear and colorless | 1.7 | 98.1 | 0.1 | 96.8 |
| 2 | T0 | clear and colorless | 1.6 | 97.8 | 0.6 | 91.7 |
| | D45 | large amount of particles | 1.7 | 97.5 | 0.8 | N/A |
| | M3 | large amount of particles | 1.8 | 97.9 | 0.3 | 97.5 |
| | M6 | large amount of particles and turbid | 1.7 | 97.8 | 0.4 | 96.7 |
| Note: T means time, D means day, and M means month. | | | | | | |

**Preparation Example 7. Filter membrane compatibility test for TGF-β receptor fusion protein preparations**

**[0190]** TGF-β receptor fusion protein (fusion protein 9) was formulated at 50 mg/ml in 10 mM citric acid-sodium citrate buffer, 80 mg/ml sucrose, 0.4 mg/ml polysorbate 80, pH 6.2. The preparations passed through a 0.22μm PES filter membrane and a PVDF filter membrane, respectively, and samples were taken at the beginning of, in the middle of and at the end of testing.

**[0191]** The experimental results are shown in Table 17. The protein content, appearance and purity analysis show that TGF-β receptor fusion protein (fusion protein 9) was stable during the contact with the filter membrane, and the preparation was compatible with both PES and PVDF filter membranes.

Table 17. Test results of compatibility with filter membranes

| Filter membrane | Concentration of protein mg/ml | SEC % | | | Non-reducing CE-SDS % | Polysorbate content mg/ml |
|---|---|---|---|---|---|---|
| | | aggregate | monomer | fragment | | |
| T0 | 50.8 | 0.8 | 98.9 | 0.3 | 98.1 | 0.46 |
| PES, primary filtrate | 51.4 | 0.9 | 98.9 | 0.2 | 98.0 | 0.46 |
| PES, medium filtrate | 49.8 | 0.9 | 98.9 | 0.3 | 98.0 | 0.46 |
| PES, final filtrate | 50.0 | 0.9 | 98.9 | 0.2 | 98.0 | 0.46 |
| PVDF, primary filtrate | 49.6 | 0.9 | 98.7 | 0.4 | 97.9 | 0.46 |
| PVDF, medium filtrate | 50.2 | 0.9 | 98.8 | 0.3 | 98.0 | 0.46 |
| PVDF, final filtrate | 50.0 | 0.9 | 98.8 | 0.3 | 97.9 | 0.45 |
| Note: T represents time. | | | | | | |

**Preparation Example 8. Lyophilization of TGF-β receptor fusion protein preparation**

**[0192]** TGF-β receptor fusion protein (fusion protein 9) preparation comprising a concentration of 50 mg/ml TGF-β receptor fusion protein (fusion protein 9), 80 mg/ml sucrose, and 0.4 mg/ml polysorbate 80 was prepared with a pH 6.2 buffer comprising 10 mM citric acid-sodium citrate. The antibody was added at 6.3 mL/vial into a 20 mL vial, and placed into a deep freezer for freeze-drying.

**[0193]** The lyophilization procedures includes pre-freezing, primary drying and secondary drying. Once the lyophilization process was over, the vial was stoppered under vacuum. The samples were reconstituted and a comparison was made between before and after freeze-drying. The results show that the reconstituted solution can maintain a favorable performance as that of the solution preparation.

Table 18. lyophilization steps of the preparations

| Parameters of lyophilization | Temperature setting (°C) | degree of vacuum (mBar) |
|---|---|---|
| pre-freezing | 5 | N/A |
| | -45 | N/A |
| primary drying | -27 | 0.1 |
| secondary drying | 25 | 0.1 |
| | 25 | 0.01 |

**Preparation Example 9. Other optional preparation compositions**

**[0194]** In addition, the present disclosure also provides other preparations of TGF-β receptor fusion protein (fusion protein 9) pharmaceutical preparations:

(1) 70 mg/ml fusion protein 9, 75 mg/ml sucrose, 0.4 mg/ml polysorbate 80, and 20 mM citric acid-sodium citrate buffer, the final pH is 6.4;

(2) 80 mg/ml fusion protein 9, 85 mg/ml sucrose, 0.5 mg/ml polysorbate 80, and 15 mM citric acid-sodium citrate buffer, the final pH is 6.2;

(3) 60 mg/ml fusion protein 9, 90 mg/ml sucrose, 0.6 mg/ml polysorbate 80, and 5 mM citric acid-sodium citrate buffer, the final pH is 6.2;

(4) 30 mg/ml fusion protein 9, 60 mg/ml sucrose, 0.3 mg/ml polysorbate 80, and 30 mM citric acid-sodium citrate buffer, the final pH is 6.3;

(5) 90 mg/ml fusion protein 9, 95 mg/ml sucrose, 0.2 mg/ml polysorbate 80, and 10 mM citric acid-sodium citrate buffer, the final pH is 6.0;

(6) 100 mg/ml fusion protein 9, 70 mg/ml sucrose, 0.1 mg/ml polysorbate 80, and 25 mM citric acid-sodium citrate buffer, the final pH is 6.5;

(7) 50 mg/ml fusion protein 9, 80 mg/ml sucrose, 0.4 mg/ml polysorbate 80, and 10 mM citric acid-sodium citrate buffer, the final pH is 7.0;

(8) 50 mg/ml fusion protein 9, 80 mg/ml sucrose, 0.4 mg/ml polysorbate 80, and 10 mM citric acid-sodium citrate buffer, the final pH is 7.5;

(9) 50 mg/ml fusion protein 9, 80 mg/ml sucrose, 0.4 mg/ml polysorbate 80, and 10 mM citric acid-sodium citrate buffer, the final pH is 5.0;

(10) 60 mg/ml fusion protein 9, 70 mg/ml sucrose, 0.5 mg/ml polysorbate 80, and 15 mM citric acid-sodium citrate buffer, the final pH is 5.5;

(11) 40 mg/ml fusion protein 9, 80 mg/ml sucrose, 0.5 mg/ml polysorbate 80, and 10 mM citric acid-sodium citrate buffer, the final pH is 6.2;

(12) 55 mg/ml fusion protein 9, 75 mg/ml sucrose, 0.3 mg/ml polysorbate 80, and 5 mM citric acid-sodium citrate buffer, the final pH is 6.0;

(13) 65 mg/ml fusion protein 9, 90 mg/ml sucrose, 0.7 mg/ml polysorbate 80, and 30 mM citric acid-sodium citrate buffer, the final pH is 7.5;

(14) 70 mg/ml fusion protein 9, 75 mg/ml sucrose, 0.8 mg/ml polysorbate 80, and 30 mM citric acid-sodium citrate buffer, the final pH is 7.0;

(15) 50 mg/ml fusion protein 9, 80 mg/ml sucrose, 0.8 mg/ml polysorbate 80, and 10 mM citric acid-sodium citrate buffer, the final pH is 7.0.

## Claims

**1.** A pharmaceutical composition comprising:

a TGF-β receptor fusion protein, and
a buffer;

wherein, the buffer is selected from the group consisting of a histidine salt buffer, a succinate buffer, and a citrate buffer.

**2.** The pharmaceutical composition according to claim 1, wherein:

the histidine salt buffer is a histidine-hydrochloric acid buffer,
the succinate buffer is a succinic acid-sodium succinate buffer,
the citrate buffer is a citric acid-sodium citrate buffer;
preferably, the buffer is a citric acid-sodium citrate buffer.

**3.** The pharmaceutical composition according to claim 1 or 2, wherein the concentration of the buffer is about 5mM to about 30mM, preferably about 5mM to about 20mM, most preferably about lOmM.

**4.** The pharmaceutical composition according to any one of claims 1 to 3, wherein the concentration of the TGF-β receptor fusion protein is about 0.5 mg/ml to about 100 mg/ml, preferably about 30 mg/ml to about 70 mg/ml, most preferably about 50mg/ml.

**5.** The pharmaceutical composition according to any one of claims 1 to 4, wherein the pH of the pharmaceutical composition is about 5.0 to about 7.5, preferably about 6.0 to about 6.5, most preferably about 6.2.

**6.** The pharmaceutical composition according to any one of claims 1 to 5, wherein:

the pharmaceutical composition further comprises a saccharide,
preferably, the saccharide is selected from the group consisting of: a trehalose and a sucrose,
most preferably, the saccharide is a sucrose.

**7.** The pharmaceutical composition according to claim 6, wherein the concentration of the saccharide is about 50 mg/ml to about 100 mg/ml, preferably about 60 mg/ml to about 90 mg/ml, most preferably about 80 mg/ml.

**8.** The pharmaceutical composition according to any one of claims 1 to 7, wherein:

the pharmaceutical composition further comprises a surfactant,
preferably, the surfactant is a polysorbate,
more preferably, the surfactant is a polysorbate 80.

**9.** The pharmaceutical composition according to claim 8, wherein the concentration of the surfactant is about 0.1 mg/ml to about 0.8 mg/ml, preferably about 0.4 mg/ml to about 0.8 mg/ml, more preferably about 0.4mg/ml.

**10.** The pharmaceutical composition according to any one of claims 1 to 9, comprising:

| | |
|---|---|
| about 0.5mg/ml to about 100mg/ml | the TGF-β receptor fusion protein, |
| about 5mM to about 30mM | citrate buffer, |
| about 50mg/ml to about 100mg/ml | sucrose, and |
| about 0.1mg/ml to about 0.8mg/ml | polysorbate 80; |

preferably, the pH of the pharmaceutical composition is about 5.0 to about 7.5; more preferably, the pH of the pharmaceutical composition is about 6.0 to about 6.5; preferably, the pharmaceutical composition comprises:

| | |
|---|---|
| about 30mg/ml to about 70mg/ml | the TGF-β receptor fusion protein, |
| about 5mM to about 20mM | citric acid-sodium citrate buffer, |
| about 60mg/ml to about 90mg/ml | sucrose, and |
| about 0.4mg/ml to about 0.8mg/ml | polysorbate 80; |

preferably, the pH of the pharmaceutical composition is about 6.0 to about 6.5; more preferably, the pharmaceutical composition comprises:

| | |
|---|---|
| about 50mg/ml | the TGF-β receptor fusion protein, |
| about lOmM | citric acid-sodium citrate buffer, |
| about 80mg/ml | sucrose, and |
| about 0.4mg/ml | polysorbate 80; |

preferably, the pH of the pharmaceutical composition is about 6.2.

**11.** The pharmaceutical composition according to any one of claims 1 to 10, wherein the TGF-β receptor fusion protein is shown as general formula (I):

Ab-L-TGF-βRII ECD (I)

wherein, the TGF-βRII ECD is a truncated form of an extracellular region of TGF-βRII;

Ab is a PD-L1 antibody or antigen-binding fragment thereof;
L is a linker sequence.

**12.** The pharmaceutical composition according to claim 11, wherein the linker sequence is shown as $(G4S)_xG$, wherein x is 3, 4, 5 or 6, preferably x is 4.

**13.** The pharmaceutical composition according to claim 11 or 12, wherein the truncated form of the extracellular region of TGF-βRII is a sequence of TGF-βRII extracellular domain with a deletion of at most 26 consecutive amino acid residues at amino terminus;

preferably, a sequence of TGF-βRII extracellular domain with a deletion of 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26 consecutive amino acid residues at the amino terminus;
more preferably, the sequence of TGF-βRII ECD is shown as SEQ ID NO: 14, 15, 16 or 17; preferably, shown as SEQ ID NO: 15.

**14.** The pharmaceutical composition according to any one of claims 11 to 13, wherein the PD-L1 antibody or antigen-binding fragment thereof comprises:

(A) HCDR1, HCDR2 and HCDR3 shown as SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3, respectively; and LCDR1, LCDR2 and LCDR3 shown as SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6, respectively; or

(B) HCDR1, HCDR2 and HCDR3 shown as SEQ ID NO: 1, SEQ ID NO: 10 and SEQ ID NO: 3, respectively, and LCDR1, LCDR2 and LCDR3 shown as SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 6, respectively.

**15.** The pharmaceutical composition according to any one of claims 11 to 14, wherein the PD-L1 antibody or antigen-binding fragment thereof comprises:

(C) a heavy chain variable region shown as SEQ ID NO: 7 and a light chain variable region shown as SEQ ID NO: 8; or
(D) a heavy chain variable region shown as SEQ ID NO: 9 and a light chain variable region shown as SEQ ID NO: 11.

**16.** The pharmaceutical composition according to any one of claims 11 to 15, wherein:

the heavy chain amino acid sequence of the PD-L1 antibody is shown as SEQ ID NO: 12 or has at least 85% identity to the amino acid sequence shown as SEQ ID NO: 12;
the light chain amino acid sequence of the PD-L1 antibody is shown as SEQ ID NO: 13 or has at least 85% identity to the amino acid sequence shown as SEQ ID NO: 13.

**17.** The pharmaceutical composition according to any one of claims 11 to 16, wherein the TGF-βRII ECD is fused to the carboxyl terminus of the heavy chain of the PD-L1 antibody through a linker sequence;
preferably, the TGF-β receptor fusion protein comprises:

(E) a fusion peptide formed by the heavy chain of the PD-L1 antibody and TGF-βRII ECD, the sequence of which is shown as SEQ ID NO: 23 or has at least 85% identity to the sequence shown as SEQ ID NO: 23, and the light chain of the PD-L1 antibody, the sequence of which is shown as SEQ ID NO: 13 or has at least 85% identity to the sequence shown as SEQ ID NO: 13; or
(F) a fusion peptide formed by the heavy chain of the PD-L1 antibody and TGF-βRII ECD, the sequence of which is shown as SEQ ID NO: 24 or has at least 85% identity to the sequence shown as SEQ ID NO: 24, and the light chain of the PD-L1 antibody, the sequence of which is shown as SEQ ID NO: 13 or has at least 85% identity to the sequence shown as SEQ ID NO: 13.

**18.** A method for preparing the pharmaceutical composition according to any one of claims 1 to 17, the method comprises:
a step of contacting the TGF-β receptor fusion protein with the buffer;

preferably, the buffer is a citric acid-sodium citrate buffer,
preferably, the concentration of the buffer is about 5mM to about 20mM, and the pH of the buffer is about 6.0 to about 6.5.

**19.** A lyophilized preparation comprising a TGF-β receptor fusion protein, which is obtained by lyophilizing the pharmaceutical composition according to any one of claims 1 to 17.

**20.** A lyophilized preparation comprising a TGF-β receptor fusion protein, which can be reconstituted to form the pharmaceutical composition according to any one of claims 1 to 17.

**21.** A reconstituted solution comprising a TGF-β receptor fusion protein, which is obtained by reconstituting the lyophilized preparation of claim 19 or 20.

**22.** An article of manufacture, comprising one or more container(s), the container comprising:

the pharmaceutical composition according to any one of claims 1 to 17, or
the lyophilized preparation comprising a TGF-β receptor fusion protein according to claim 19 or 20, or
the reconstituted solution comprising a TGF-β receptor fusion protein according to claim 21.

**23.** Use of any one selected from the following in the preparation of a medicament:

the pharmaceutical composition according to any one of claims 1 to 17, or the lyophilized preparation comprising a TGF-β receptor fusion protein according to claim 19 or 20, or the reconstituted solution comprising a TGF-β receptor fusion protein according to claim 21, or the article of manufacture according to claim 22;

preferably, the medicament is used for treating or inhibiting disease(s) or disorder(s) related to tumor cell proliferation or tumor cell metastasis;
more preferably, the disease(s) or disorder(s) is/are a tumor;
more preferably, the disease(s) or disorder(s) is/are selected from the group consisting of: head and neck cancer, glioblastoma, glioma, nasopharyngeal carcinoma, thyroid cancer, lung cancer, myeloma cancer, myelodysplastic syndrome, neuroendocrine cancer, lymphoma, leukemia, melanoma, basal cell cutaneous carcinoma, squamous cell cutaneous carcinoma, dermatofibrosarcoma protuberans, Merkel cell carcinoma, sarcoma, mesothelioma, gastric cancer, liver cancer, pancreatic cancer, kidney cancer, bladder cancer, colorectal cancer, breast cancer, endometrial cancer, uterine cancer, cervical cancer, ovarian cancer, prostate cancer and testicular cancer; the lung cancer is selected from the group consisting of: small cell lung cancer and non-small cell lung cancer.

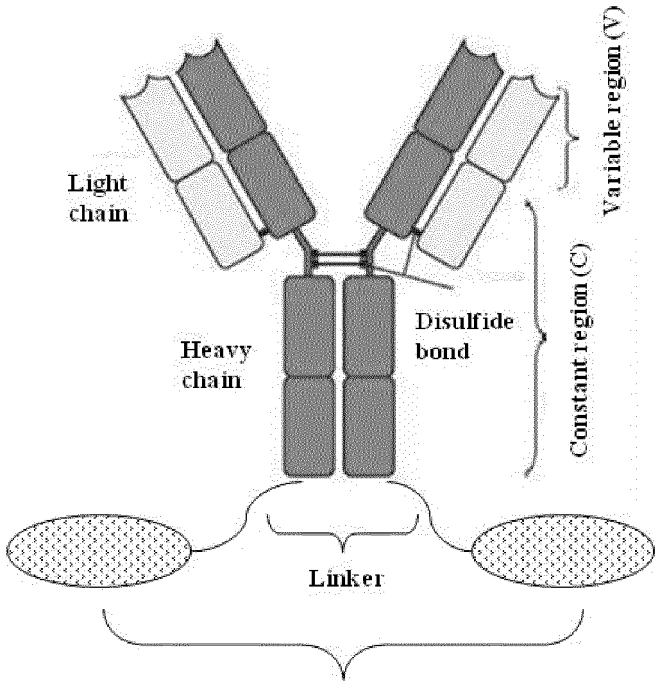



Figure 1

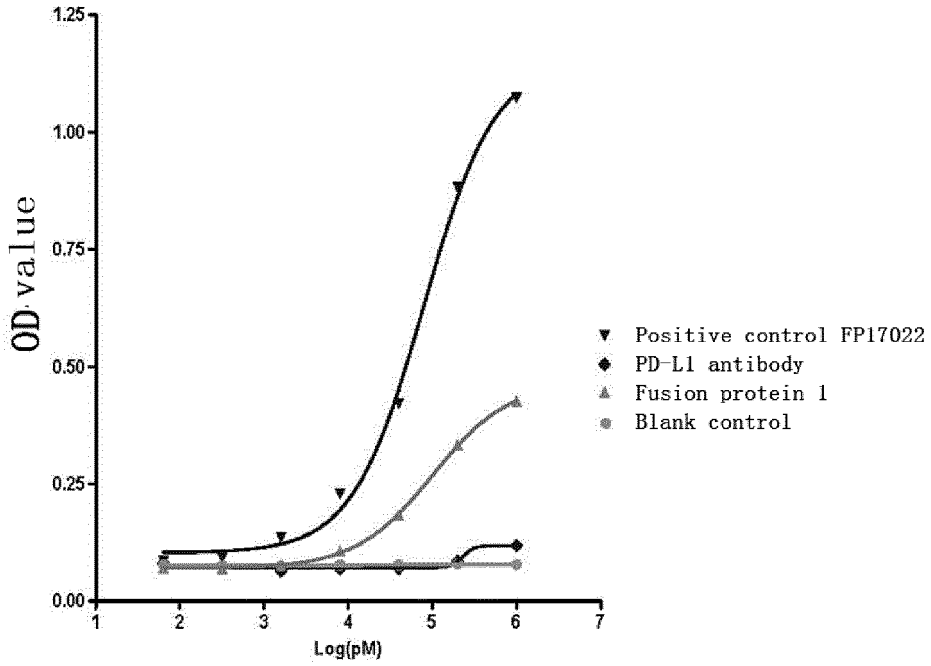
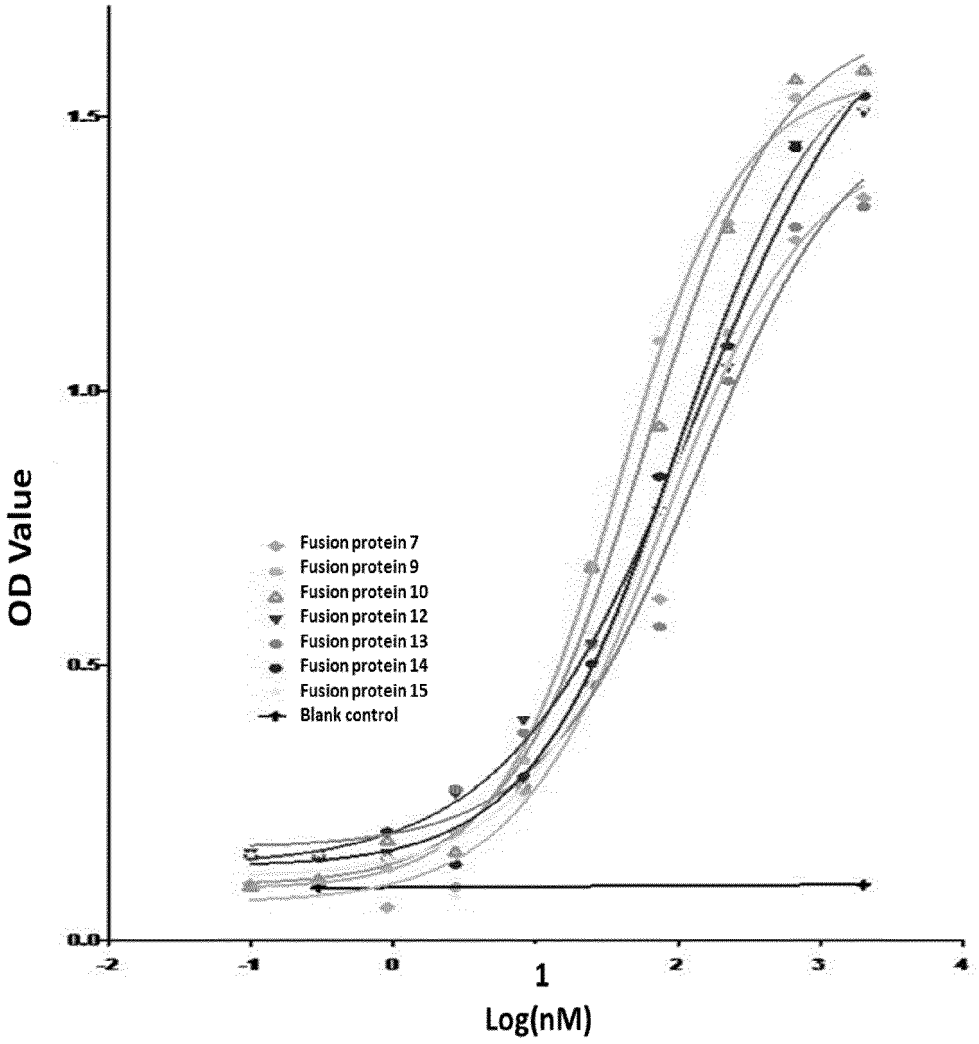



Figure 2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0.0
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-1
0
1
2
3
4
Log(nM)
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Fusion protein 9
Fusion protein 10
Fusion protein 12
Fusion protein 13
Fusion protein 14
Fusion protein 15
Blank control

**Figure 3**

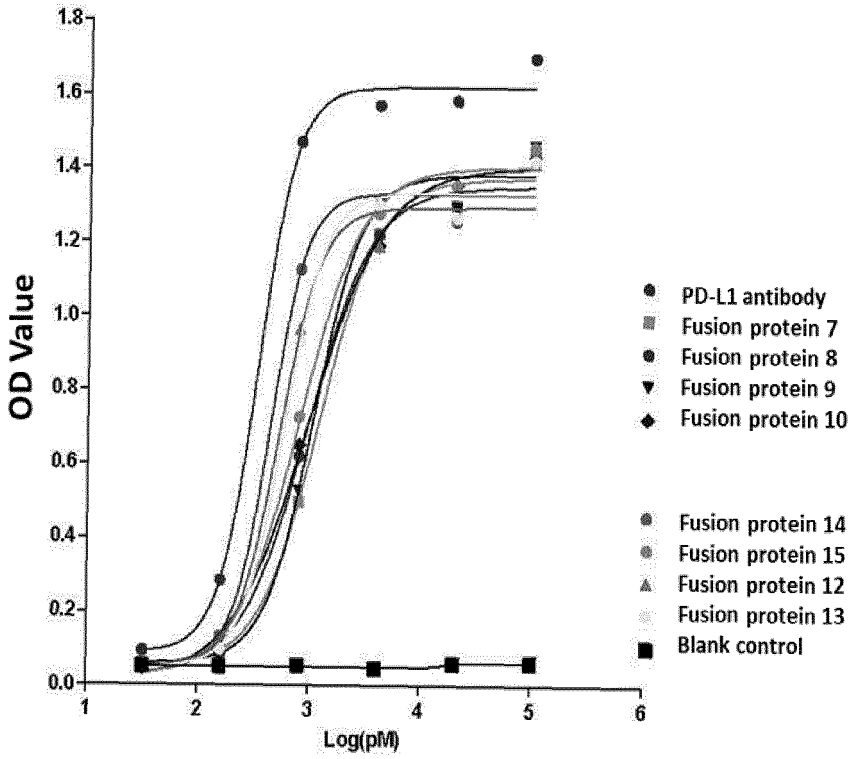



Figure 4

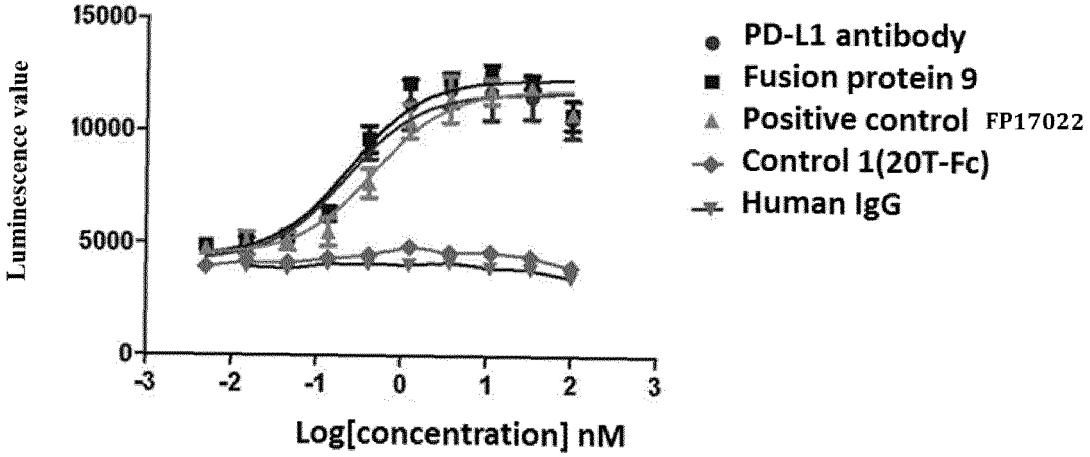



Figure 5

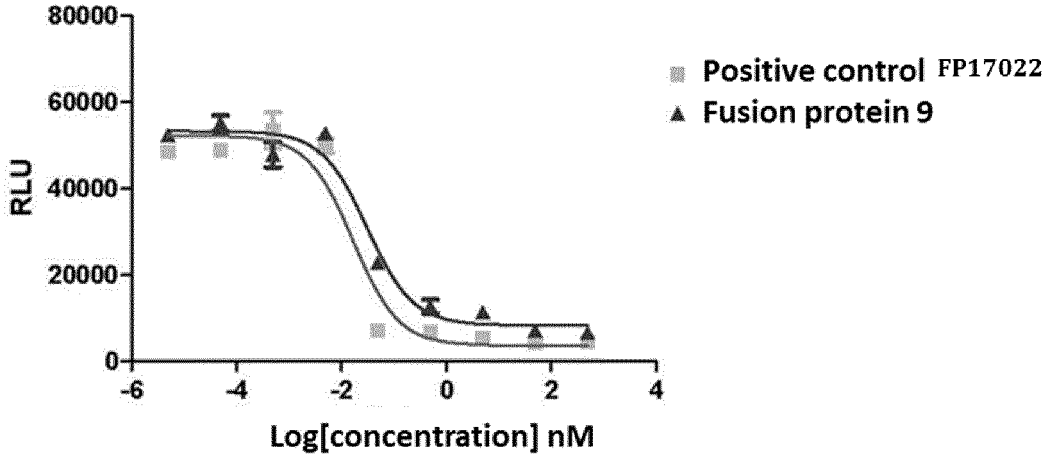



Figure 6

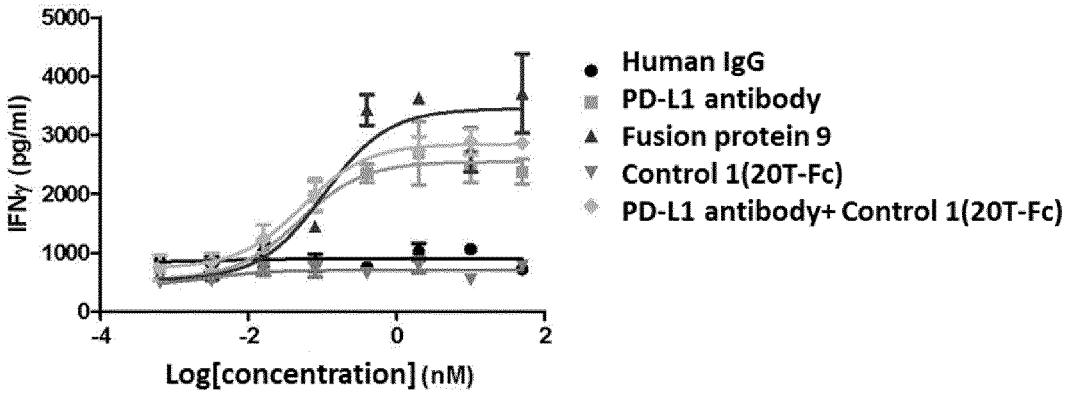
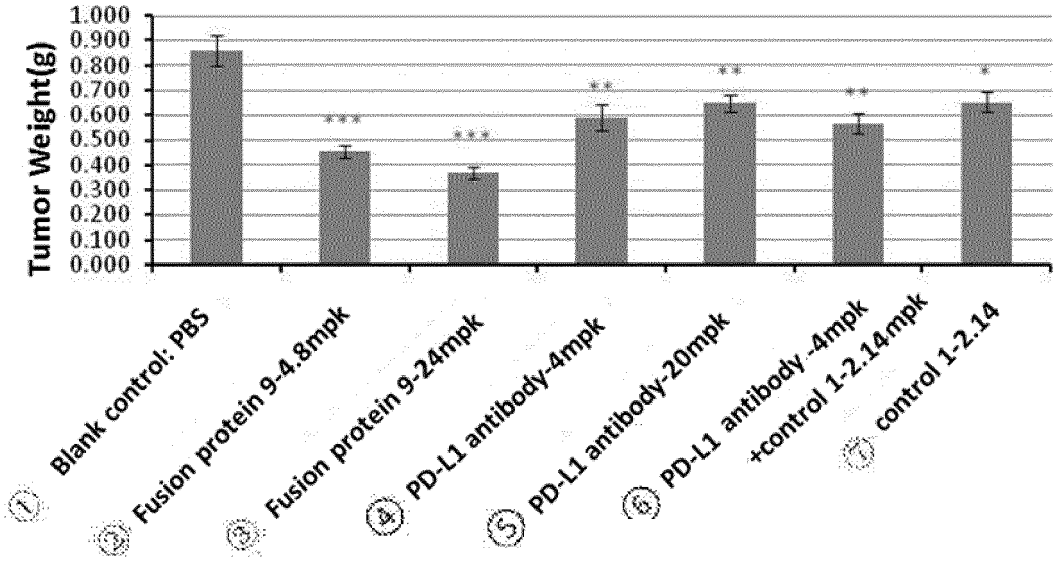



Figure 7


Tumor Weight(g)
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0.900
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0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
***
***
**
**
**
*
① Blank control: PBS
② Fusion protein 9-4.8mpk
③ Fusion protein 9-24mpk
④ PD-L1 antibody-4mpk
⑤ PD-L1 antibody-20mpk
⑥ PD-L1 antibody-4mpk +control 1-2.14mpk
⑦ control 1-2.14

**Figure 8**

**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/CN2019/116593**

**A. CLASSIFICATION OF SUBJECT MATTER**

A61K 38/16(2006.01)i; A61K 38/17(2006.01)i; C07K 19/00(2006.01)i; A61P 35/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K; C07K; A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNABS, CPRSABS, SIPOABS, DWPI, CNTXT, WOTXT, EPTXT, USTXT, CNKI, 百度学术搜索, BAIDU SCHOLAR SEARCH, WEB OF SCIENCE, PubMed, GenBank, 中国生物序列检索数据库, CHINA BIOLOGICAL SEQUENCE SEARCH DATABASE: TGF-β受体, 融合蛋白, 组氨酸盐缓冲液, 琥珀酸盐缓冲液, 枸橼酸盐缓冲液, 海藻糖, 蔗糖, 表面活性剂, 聚山梨醇酯, 胞外区, PD-L1抗体, transforming growth factor (TGF)beta receptor, TGF-βreceptor, fusion protein, fusion polypeptide, Ab-L-TGF-βRII ECD, PD-L1 antibody, 基于SEQ ID NOs: 1-17, 23, 24的序列检索, search based on SEQ ID NOs: 1-17, 23, 24

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | CN 101397343 A (CHENGDU KANGHONG BIOTECHNOLOGY CO., LTD.) 01 April 2009 (2009-04-01)<br>see claims, and description, page 3 | 1-23 |
| A | WO 2015183943 A3 (TRUSTEES OF BOSTON UNIVERSITY) 28 January 2016 (2016-01-28)<br>entire document | 1-23 |
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☐ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| **06 February 2020** | **26 February 2020** |
| Name and mailing address of the ISA/CN<br>**China National Intellectual Property Administration**<br>**No. 6, Xitucheng Road, Jimenqiao Haidian District, Beijing 100088**<br>**China** | Authorized officer |
| Facsimile No. **(86-10)62019451** | Telephone No. |

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**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/CN2019/116593**

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed:
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      ☐ on paper or in the form of an image file.
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**INTERNATIONAL SEARCH REPORT**
**Information on patent family members**

International application No.
**PCT/CN2019/116593**

| Patent document cited in search report | Publication date (day/month/year) | Patent family member(s) | Publication date (day/month/year) |
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